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Phylogenetic Inference from 18S rRNA Gene Sequences of Horseshoe Crabs, *Tachypleus gigas* between Tanjung Dawai, Kedah & Cherating, Pahang, Peninsular Malaysia

Ismail, N., & Sarijan, S

Abstract**—**The phylogenetic analysis using the most conservative portions of 18S rRNA gene revealed the phylogenetic relationship among the two populations where DNA divergence showed that the nucleotides diversity value were -0.00838 for the Tanjung Dawai, Kedah and -0.00708 for the Cherating, Pahang populations respectively. The net nucleotide divergence among populations (Da) was -0.0073 indicating a low polymorphism among the populations studied. Total number of mutations in the Tanjung Dawai, Kedah samples was higher than Cherating, Pahang samples, which are 73 and 59 respectively while shared mutations across the populations were 8, and reveal the evolutionary in the genome of Malaysian *T. gigas*. The tree topology of both populations inferred using Neigbour-joining method by comparing 1791 bp of partial 18S rRNA sequence revealed that *T. gigas* haplotypes were clustered into seven clades, suggesting that they are genetically diverse among populations which derived from a common ancestor.

Keywords**—**Horseshoe crabs, *Tachypleus gigas*, 18S rRNA gene sequences, phylogenetic analysis

I. INTRODUCTION

HORSESHOE crab or also known as king crab is the only primitive marine invertebrate which widely distributed on earth. They have been classified in Phylum Arthropoda; Subclass Xiphosura and phylogenetically more related to arachnids than to crustaceans [8], [13]. Horseshoe crabs are often called as a unique living fossil which has been in continuously present for 550 million years of evolution without modification apparent on their morphology [7]. Their exoskeleton is very similar to a fossil specimen (*Mesolimulus walchi*) found in the Jurrasic deposits [14], [7]. Three species from the Subfamily Tachypleinae are *Tachypleus gigas*, *T. tridentatus*, and *Carcinoscorpius rotundicauda*. These species inhabit the coasts of Indo-Pacific [4] and has been clustered as Asian horseshoe crabs. *T. gigas* can usually be found from the Indian shore to the Southeast Asia region and disperse to the south-western part of Lantau Island, Hong Kong. In this country, *T. gigas* occur around Peninsular Malaysia and small amount in Borneo Island.

Ismail, N & Sarijan, S are with the Department of Biological Sciences, Faculty of Science and Technology, University Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia (phone: 6096683240; e-mail: noraznawati@umt.edu.my).

This study was conducted to assess the relationships of *T. gigas* between two populations in Peninsular Malaysia. We chose these two localities because the horseshoe crabs in both populations were exploited for delicacies and are exported to neighbour country. Here, the 18S rRNA gene sequences of this species were obtained in order to investigate their phylogeny. The nucleotides divergence between populations was also successfully examined, thus reveals the evolutionary occurred in Malaysian *T. gigas*.

II. MATERIAL AND METHOD

A. Collection of Horseshoe Crab Samples

A total of twenty individuals of adult horseshoe crab, *T. gigas* were collected from different shorelines in Peninsular Malaysia. Two sites were selected namely Tanjung Dawai, Kedah (5° 41' 0" N, 100° 22' 0" E) and Cherating, Pahang (4° 6' 0" N, 103° 23' 0" E) which is located on the western and eastern coast of Peninsular Malaysia respectively (Figure 1). Each location has ten individuals and the distance between the two locations is approximately 971 km along the shoreline. Live samples for each population were purchased from the fishermen and gill covers were excised soon after the horseshoe crabs were brought back to the laboratory.

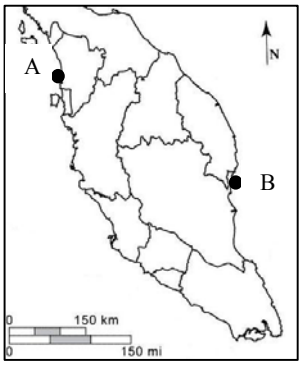

Fig. 1 Sampling sites of Peninsular Malaysia coasts. The west-coast site A is located at Tanjung Dawai, Kedah and the east-coast site B is located at Cherating, Pahang

B. Isolation and standardization of genomic DNA concentration

Gill covers of horseshoe crab were wiped using 70% alcohol to prevent contamination and then placed into a sterilized 1.5ml microcentrifuge tube. Genomic DNA for each individual

was extracted using the prescribed protocol of DNeasy® Blood & Tissue Kit (Qiagen, USA). According to Giacomazzi et al. (2004), the DNA extraction kit can improve the PCR sensitivity and prevent the amplification of non-specific PCR products. The quantity of DNA was measured by the absorbance readings at 260 nm using UV Biophotometer (Eppendorf, Germany). The purity of DNA was estimated by calculating the ratio of absorbance at 260 nm and 280 nm. The DNA samples were concentrated to 100ng/µl as the standard concentration. The concentration of each DNA sample was standardized to 100ng/µl by dilution with appropriate amount of sterile distilled water.

C. Amplification and Sequencing of 18S rRNA Gene Fragment

The 18S gene of *T. gigas* was amplified with eukaryotic-specific 18S rRNA gene primers (2–22F, 5'–CCGTCGACGAGCTCAACCTGGTTGATCCTGCCAGT–3' and 1864-1842R, 5'–CCCGGGTACCAAGCTTGATCCWTCTGCAGGTTCACCTAC–3') in a PCR reaction containing 2.5 µl of 10× *Taq* Buffer (Fermentas), 2.5µl of dNTP mix (2mM, Fermentas), 1µl of each primer, 1 U of *Taq* DNA polymerase (Fermentas) and 50ng/µl of genomic template DNA in a final volume of 25.0µl. The amplification program was started with one initial denaturation step of 95°C for 180 seconds; 35 cycles consisting of denaturation at 95°C for 60 seconds, primer annealing at 59.1°C for 150 seconds and elongation at 72°C for 180 seconds. The final elongation step was extended for 420 seconds. The amplification reaction was performed by using a MasterCycler gradient (Eppendorf, Germany). The 18S rRNA was amplified by PCR and approximately 1800bp segment of the 18S rDNA gene was purified by following the protocol of QIAquick Gel Extraction Kit (Qiagen, USA). The segment was cloned in *E. coli* cell, pGEM®-T Easy Vector System (Promega, USA) and sequenced.

D. Phylogenetic Analysis of 18S rRNA gene Fragment

The obtained gene fragment was aligned with the different gene fragments of the various horseshoe crabs species by using BLAST (Basic Local Alignment Search Tool) search (http://www.ncbi.nlm.nih.gov/BLAST) to investigate the regions of similarity between sequences. Comparative sequence analysis of 18S rRNA gene clones was examined by using neighbour-joining method to develop a phylogenetic tree. This tree diagram was viewed by using MEGA 4 software after computed in PHYLIP (Ver 3.69) software to generate the out-tree file. The DNASP (DNA Sequence Polymorphism) software was then used to analyze the DNA divergence between populations in which the Kimura's two-parameter distance was applied.

III. RESULTS

DNA sequencing results reveals a 1832 bp fragment of the partial 18S rRNA which was successfully isolated from the *T. gigas* genomic DNA. The edited nucleotide sequence of the *T. gigas* partial 18S rRNA gene was then used as a query in the public available BLAST database (Basic Local Alignment Search Tool) search (http://www.ncbi.nlm.nih.gov/BLAST). The alignment shows the high homology of nucleotide sequence to 18S rRNA gene of two different species of horseshoe crabs, *C. rotundicauda* (99 %) (accession number U91491) and *L. polyphemus* (98 %) (accession number L81949 and U91490) but none for *T. gigas.* The tree-view depicted that the partial 18S rRNA gene of samples studied shared the sister taxon with 18S rRNA gene isolated from the *C. rotundicaud*a with a bootstrap value of 99 %.

In this study, the isolated genes were cloned and registered into GenBank with the accession number GU827391 (as our phylogeny reference), HQ876469, HQ876470, HQ876471, HQ876472, HQ876473, HQ876474, HQ876475, HQ876476, HQ876477, HQ876478, HQ876479, HQ876480, HQ876481, HQ876482, HQ876483, HQ876484, HQ876485, HQ876486, HQ876487, HQ876488 as references for the identification of *T.* gigas phylogeny. The multiple alignments for different samples of both populations were carried out and the overall nucleotide sequence similarity was 99 % (data not shown). These sequences were also analyzed for phylogenetics particularly on their DNA divergence between populations. Kimura's two parameter distances analysis was applied after completely excluding sites with gaps from the analysis. It was found that the populations of Tanjung Dawai, Kedah and Cherating, Pahang showed extremely low nucleotide diversity (Table 1). The number of mutations in the Tanjung Dawai, Kedah and Cherating, Pahang populations were 73 and 59 respectively while shared mutation across populations was 8. This indicated that the changes have occured in the DNA sequence of the 18S gene. Net nucleotide diversity (π) in the Tanjung Dawai, Kedah and Cherating, Pahang population were -0.00838 and -0.00708 respectively thus shows that the low degree of polymorphisms within both populations studied.

TABLE I
DNA DIVERGENCE BETWEEN POPULATIONS

DNA Divergence	Tanjung Dawai, Kedah	Cherating, Pahang
Number of sequences	10	10
Total sites in nucleotide sequence	1791	1791
Total number of mutations	73	59
Nucleotide diversity	-0.00838	-0.00708
Shared mutations: 8		
Net nucleotidedivergence: -0.00773		

The phylogenetic tree of both populations was inferred using the Neighbour-joining method (Saitou & Nei, 1987) and projected using PHYLIP (Ver 3.69) software. The tree topology obtained was quantified by the bootstrap method with 1000-iterations. Figure 2 represents the phylogenetic relationships of *T. gigas* in the Tanjung Dawai, Kedah and Cherating, Pahang populations. In this analysis, the phylogeny of *T. gigas* in both populations was constructed by comparing the 1791 bp DNA sequence of the 18S rRNA gene and the total sites of sequence were determined after excluding the alignment gaps of each individual. According to the

polygenetic tree generated, *T. gigas* are not distinctly separated into two populations but they are divided by the clade groups. The branches of the tree represent the pathway that traces the evolutionary history of *T. gigas* lineages which were connected through polytomy.

The generated Neighbour-joining tree indicated that the Malaysian *T. gigas* haplotypes were clustered into seven clades (I-VII) based on their sisters' pair. A pair of taxa is generally known as sisters, for example in clade I, C15 and C6 are sisters as are in clade V, T25 and T22. Individuals in clade II is a sister taxon to the clade I, which were share a common ancestor at the node that joins them together. The first group, clade I, included two of the ten haplotypes found in the Cherating, Pahang samples. Meanwhile five haplotypes from the Tanjung Dawai, Kedah samples made up another two groups namely clade IV and V (Figure 2). The other haplotypes was clustered into four groups according to the connected branches and nodes of the tree namely clade II, III, VI and VII which is a mixture of *T. gigas* from both populations.

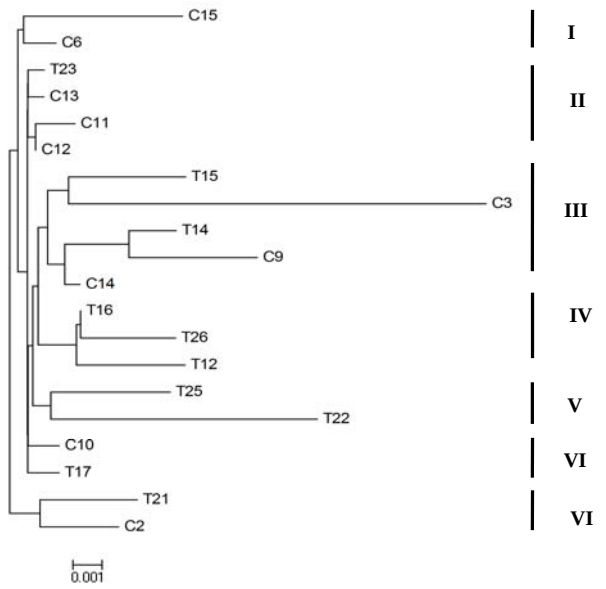

Fig. 2 Phylogenetic inference derived from distance analysis of partial 18S rRNA gene of *T. gigas* nucleotide sequence. The scale bar indicates an evolutionary distance of 0.001 nucleotides per position in the sequence.

IV. DISCUSSION

The analysis of nuclear rDNA, which encodes rRNA gene, is commonly used in assessing phylogenetic relationships among taxa [5], [3]. In this study, the most conservative portions of the 18S rRNA gene were successfully used to investigate the phylogenetic relationships, evolutions, and genetic diversity of Malaysian *T. gigas*. More than 1800 nucleotides sequence were successfully isolated from the *T. gigas* genome representing the conservative portions of the 18S rRNA molecule. Results showed that the homology sequences of *T. gigas* (accession number GU827391) are phylogenetically more related to *C. rotundicauda* (accession number U91491) with the bootstrap value of 99%. This is in agreement with the previous research conducted by Xia (2000) which grouped these two species as a monophyletic taxon based on the most-parsimonious tree (MP tree) from different molecular techniques such as 16s rRNA gene, cytochrome oxidase I (COI), and amino acid sequences of nuclear gene. The sequence alignment also revealed that *L. polyphemus* (accession number L81949) is the sister taxon of *C. rotundicauda* (accession number U91491) and the Malaysian *T. gigas*.

In this study, the Kimura two-parameter (K2P) distance was used to estimate nucleotide diversity of both populations through evolutionary base substitutions comparison of nucleotide sequences. As a result, DNA divergence showed that nucleotide diversity in the Tanjung Dawai, Kedah and Cherating, Pahang population was extremely low indicating the low polymorphism in both populations studied. This would imply that both populations were small in size. Small and isolated populations are more vulnerable to extinction from their living sites.

This finding is important since both locations were also specially selected for the lysate study in Malaysia and due to the continuous harvesting of *T. gigas* as a delicacy. In this study, it was found that, the total number of mutations in the Tanjung Dawai, Kedah population was higher than Cherating, Pahang. This would imply that the *T. gigas* genome in both populations have evolved throughout evolution. According to [10], some of mutations would spontaneously improve the fitness of organisms and become fixed in a population. Improved fitness is essential for a better survival potential and reproduction of the animals [12]. In this study, it would imply that the Tanjung Dawai, Kedah population would be more fit than of the Cherating, Pahang population. In addition, both populations are nearer to the fishermen settlement and the occurrence of the oil spill from the fishing boat and trading ship into ocean is might be one of the factors to the mutation. The high mutation in Tanjung Dawai, Kedah also might be due to the narrow channel of Straits of Malacca as compared to wider ocean in Cherating, Pahang. Here, it could suggest that the difference of water parameters may have caused to the declination and the mutation level of *T. gigas* in both shorelines.

As for the Neighbour-joining tree, the Malaysian *T. gigas* was genetically diverse among the two populations. The individuals were genetically more closely related to each other although some made up their own clades. This would imply that the diversity in both populations were evolutionarily from a common origin. The distance between Tanjung Dawai, Kedah and Cherating, Pahang was approximately less than 971 km along the shoreline and therefore, any assumptions of outbreeding activity between the populations would be totally rejected as movement and the possible home range size of horseshoe crabs are constrained to 641 m^2 and 614 m^2 for breeding purposes [6]. Several studies on their movement patterns have also conducted on *L. polyphemus* as examples: the maximum distance recorded on this species in the Great Bay Estuary, New Hampshire, USA was 9.2 km [9], and a 17-years tagging study on 30 000 individuals from New York to

Delaware and Chesapeake Bays documented 14 individuals were moved in the range of 104 – 265 km [11]. Reference [2] was successfully revealed the maximum distance of horseshoe crab according to their sex: 493.74 km for female and 363.7 km for male thus summarized that the female could move farther than of male horseshoe crab.

Apart from that, long distance migration of larval stages of *T. gigas* to another place for growing process until mature state is also impossible. According to previous study, larval horseshoe crabs are strongly dependent on nearshore, thus farther dispersal from spawning site to another along inshore continental shelf is less recorded [1]. This sequence-based molecular marker was successfully elucidates the phylogenetic relationship and evolution of *T. gigas* from Cherating, Pahang and Tanjung Dawai, Kedah. The generated 18S rRNA tree indicated that *T. gigas* found in the Malaysian seafloor originated from a common ancestor. This research has also provided the evidence of evolutionary distance among individuals is less for both populations studied.

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