ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 10 (3): 298-302, 2011
ISSN 1682-296X / DOI: 10.3923/biotech.2011.298.302

Genetic Structure and Haplotype Diversity of *Tachypleus gigas* Population along the West Coast of Peninsular Malaysia-Inferred through mtDNA AT Rich Region Sequence Analysis

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Abstract: A detailed investigation was carried out to determine the genetic structure and haplotype diversity of Malaysian horseshoe crab (*Tachypleus gigas* [Muller, 1785]) distributed along the west coast of Peninsular Malaysia. Mitochondrial DNA (AT rich region = 369 bp) analysis showed that *T. gigas* had 13 haplotypes along the Malaysian west coast of which 4 were unique to Selangor samples while 3 were unique to Johor sample and 1 each were unique to other two stations respectively. Highest haplotype diversity (h) was observed among the Selangor samples (0.873±0.071) followed by Langkawi, Johor and Kedah samples with 0.833±0.222, 0.752±0.066 and 0.733±0.155 values, respectively. Over all haplotype diversity of *T. gigas* in west coast of Malaysia was observed to be 0.797±0.129. Pair wise haplotype frequency (F_{ST}) value were statistically significant ($p<0.05$) for all the groups except for Langkawi/Kedah samples indicating higher gene flow (Lower haplotype diversity) among these two populations. Average nucleotide diversity (π) was higher in Selangor samples (0.0083±0.001) followed by Johor (0.0063±0.0011) and it was almost similar in Langkawi (0.0045±0.0012) and Kedah (0.0040±0.0008) samples which indicated higher polymorphic sites in Selangor and Johor samples while it was lower in Langkawi and Kedah samples. In addition phylogenetic analysis clearly clustered *T. gigas* samples from *T. tridentatus* samples indicating good phylogenetic signals in mtDNA AT rich region. Overall, findings from this study have important implications for proper management and conservation of this living fossil along the west coast of Peninsular Malaysia.

Key words: Haplotype diversity, nucleotide diversity, mtDNA AT rich region, *Tachypleus gigas*, genetic conservation

INTRODUCTION

Horseshoe crabs are one of the interesting groups of organism maintaining their genetic structure virtually unchanged over millions of years (Kamaruzzaman *et al*., 2011; John *et al*., 2010b). Their limited distribution, restricted gene flow in the population and higher stress tolerance ability would have played a key role in their emergence over time (Smith *et al*., 2009a; Smith *et al*., 2009b). *Tachypleus gigas*is one of the four extant species of horseshoe crab found in shallow water in South East Asia at depths of up to 40 m (130 ft). In Malaysia, their distribution was recorded in both the coasts including Borneo island (Kassim *et al*., 2008; Smith *et al*., 2009c; Smith *et al*., 2009d). Members of this species generally inhabit a cove or bay that is protected from surf (Almendral and Schoppe, 2005). Same like *T. tridentatus*, they molt at least 13 times (for male) and 14 times (for female) as they grow from the larval stage to sexual maturity. This period exceeds 10 years for captive breeding from an egg (Harada, 2003).

In recent decades, various molecular tools have been widely used in variety of generic diversity studies, population structure prediction including their phylogeny and genetic distance analysis. Among which mitochondrial DNA (mtDNA) sequencing analysis is widely being used for a range of molecular level predictions (John *et al*., 2010a). It was also evident from recent studies that molecular genetic analysis is a powerful tool for investigating genetic differentiation within a population, genetic structure and diversity throughout the history of a population (Ward *et al*., 2005). Analysis of populations of the American horseshoe crab (*Limulus polyphemus*) along the eastern coast

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of North America using several genetic markers detected a major genetic ''break'' between the northern and southern populations along the Florida state by using allozyme (Giribet *et al*., 2001), mitochondrial DNA restriction fragment length polymorphism (RFLP) analysis (Pierce *et al*., 2000) and DNA microsatellite (King and Eackles, 2004). Sequence analyses of mitochondrial Cytochrome C Oxidase subunitI (COI) suggested that there has been limited gene flow between these populations (Pierce *et al*., 2000).

The mtDNA AT-rich region is a highly variable, non coding region that is useful for phylogeographic studies and population genetic surveys, although the high AT content poses technical and analytical problems (Vila and Bjorklund, 2004). Research had been carried out to predict the population structure of tri spine horseshoe crab (*T. tridentatus*) using mtDNA AT rich region as a marker gene (Yang *et al*., 2007). Due to the lack of genetic information on *T. gigas* population along the Malaysian coast, present work was initiated to investigate genetic structure and genetic diversity of Malaysian horseshoe crab (*T. gigas*) using mtDNA AT rich region as a reference gene.

MATERIALS AND METHODS

Study site and sample collection: A total of 4 sampling sites were identified along the west coast of Peninsular Malaysia and 39 samples were collected from the nesting beaches of *T. gigas* (Johor = 18; Selangor = 11; Langkawi = 4 and Kedah = 6) during 2008 (Fig. 1). Samples were immediately iced prior to laboratory analysis. Sampling sites were geographically divided into 3 distinct regions, 1. South Peninsular Malaysia (SPM-Johor site), 2. West Peninsular Malaysia (WPM-Selangor site) and 3. North Peninsular Malaysia (NPM-Langkawi and Kedah sampling sites) (Table 1). Samples were dissected out using sterilized scissors and 2×2cm soft muscle tissue were excised and preserved in 95% ethanol (John *et al*., 2010a).

DNA extraction, PCR and DNA sequencing: Salting out procedure was adopted for precise and quick DNA isolation from horseshoe crab samples (John *et al*., 2010b). The complete AT rich region of mtDNA was amplified by a pair of primers, Hb-trna (5'-GAGCCCAATAGCTTAAATTAGCTTA-3') and Hb-12S (5'- GTCTAACCGCGGTAGCTGGCAC-3') (Yang *et al*., 2007). Amplification reaction was conducted in 50 μL buffer supplied with the enzyme and under the conditions recommended by the manufacturer (Invitrogen, Germany). Each 50 μL volume contained 50 mM KCl, 10 mM Tris (pH 9), 3 mM $MgCl_2$ 0.2 mM each dNTP, 0.04 mM each primer, 0.033 units of Tag polymerase, 1 μL DMSO and

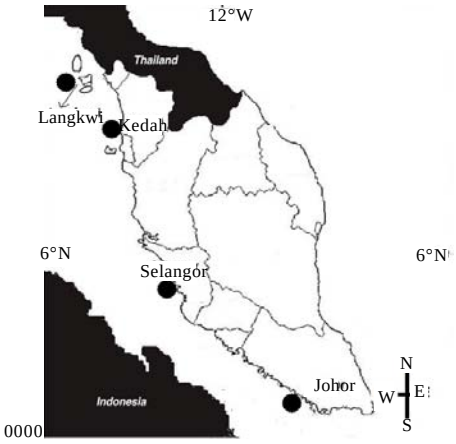

Fig. 1: Location of the sampling area

Table 1: Detailed information of the sampling location and geographical information

Geographical region	Sampling site	Sample ID	Site locations (GPS reading)
SPM	Johor	JOH	N 01° 54.756' E 102° 39.448'
WPM	Selangor	SEL	N 02° 36.753' E 101° 41.064'
NPM	Langkawi	LAN	N 06° 18.578' E 099° 43.331'
NPM	Kedah	KED	N 05° 39.681' E 100° 19.907'

SPM: South peninsular Malaysia, WPM: West Peninsular Malaysia, NPM: North Peninsular Malaysia

50 ng of mtDNA. The thermocyclic conditions for PCR included the initial denaturation at 94°C for 1 min, five cycles of 94°C for 30 sec, annealing at 45°C for 40 sec and extension at 72°C for 1 min, with a final extension at 72°C for 10 min, followed by indefinite hold at 4°C. Following PCR, about 10 μL of PCR product with 2 μL of bromo thymol blue were added to 2% agarose gel, prepared with 2.5 μL of 1% Ethidium bromide and electrophorized at 90 V until the dye moved for 6 cm in the gel. The gel was moved to gel doc system for viewing the amplicons with the aid of UV trans-illuminator. Final PCR product was sequenced using ABI 3730×1 sequencer and obtained chromatogram was edited via ABI sequence scanner software 1.0v.

Sequence and population-genetic analyses: All data analyses were analyzed using Arlequin 3.0v for a Macintosh platform (Excoffier and Schneider, 2005). Unique haplotypes and all transitions and transversions were counted. Haplotype diversity (h), nucleotide diversity (Shimatani, 1999) and their standard errors were calculated. Pairwise F- statistics (F_{ST}) were calculated as genetic distances based on pair wise differences between populations using DnaSP software 4.50.3v (Rozas *et al*., 2003). An indirect estimate of gene flow was calculated based on the Eq. 1:

$$N_em = 0.5\times[(1/F_{ST}) - 1] \quad (1)$$

where, N_e is the effective number of females and m is the migration rate. Percentage of AT was calculated using Bio edit software (Hall, 1999) and Transition:Transversion (Ti:Tv) ratio was calculated using MEGA 4.0 (Tamura *et al*., 2007).

RESULTS AND DISCUSSION

Data analysis proved that the AT rich region undergoes mainly transition mutation (Ti) and the samples from west coast of Malaysia had average Ti: Tv ratio of 2:0. The observed mean AT content in the controlled region (mtDNA AT rich region) was 86.37% in the *T. gigas* samples. Highest haplotype diversity (h) was observed among Selangor samples (0.873±0.071) followed by Langkawi, Johor and Kedah samples with 0.833±0.222, 0.752±0.066 and 0.733±0.155 values respectively. Over all haplotype diversity of *T. gigas* in west coast of Malaysia was observed to be 0.797±0.129 (Table 2). This observation suggested that the Selangor and Langkawi population of this species might have been formed recently and that the dispersal rate has been relatively low, leading to the formation of genetically distinct populations. Mean nucleotide diversity (π) was higher in Selangor samples (0.0083±0.001) followed by Johor (0.0063±0.0011) and it was almost similar in Langkawi (0.0045±0.0012) and Kedah (0.0040±0.0008) samples which indicates higher polymorphic sites in Selangor and Johor successfully used as a molecular marker for species identification and for determination of population genetic structure in a wide variety of aquatic taxa (Ward *et al*., 2005; Thorpe *et al*., 2000; Quan *et al*., 2001). It is also to be noted that MtDNA gives a better estimate of genetic differentiation than nuclear markers since it is approximately four fold more sensitive (Lorenz *et al*., 2005).

A total of 13 haplotypes were identified in *T. gigas* samples from west coast, of which 4 haplotypes were unique to Selangor samples (TG6,7,8 and 9) and 3 each were unique to Johor samples (TG2,4 and 5) and 1 each were unique other 2 stations Langkawi (TG11) Kedah (TG13). This observation showed that SW Malaysian horseshoe crab population had higher haplotype diversity samples while it was lower in Langkawi and Kedah samples. Similar observation was reported by Yang *et al*. (2007) where he observed *T. tridentatus* population from closer geographical area of Taiwan coastal waters showed almost similar level of nucleotide diversity. Present study also proved the competence of mtDNA region in various molecular marker studies. Hence it was evident that Mitochondrial DNA (mtDNA) analysis could be which would ultimately lead to restricted gene flow among

Table 2: Localities and molecular characters in *T. gigas* mtDNA AT-rich region

Sampling site	No. of samples	(A+T)%	Ti:Tv	Nh	h±SD	π±SD
Johor	18	86.40	2:0	5	0.752±0.066	0.0063±0.0011
Selangor	11	85.90	3:0	6	0.873±0.071	0.0083±0.0010
Langkawi	4	86.70	2:0	3	0.833±0.222	0.0045±0.0012
Kedah	6	86.50	1:0	3	0.733±0.155	0.0040±0.0008
Average	n = 39	86.37	2:0	19	0.797±0.129	0.0058±0.001

Sample size (n), Nucleotide content (AT%), Number of substitutions (Ti, transition: Tv, Transversion), Number of haplotypes (Nh), Haplotypes diversity (h) and Nucleotide diversity (π)

Table 3: Variable sites found in a fragment of AT-rich region of *Tachypleus gigas* and their distribution in the population

	Nucleotide positions																Populations			
	6	7	9	1 0	1 2	1 3	1 3	1 5	1 5	1 8	1 9	1 9	2 0	2 8	2 9	3 0	JOH	SEL	LAN	KED
Haplotypes	6	6	6	1	2	1	8	1	4	2	4	6	9	6	9	8	n = 18	n = 11	n = 4	n = 6
TG1	G	G	T	A	A	T	C	C	A	T	A	A	G	A	T	C	0.389	0.273		
TG2	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	T	0.333			
TG3	*	A	C	*	*	*	*	*	*	*	G	*	*	G	*	*	0.111	0.09		
TG4	A	*	*	G	*	*	*	*	*	*	*	*	*	*	*	T	0.111			
TG5	*	A	*	*	G	*	*	*	*	*	*	G	*	*	*	*	0.056			
TG6	*	A	*	*	G	*	*	*	*	*	G	*	*	*	*	*		0.273		
TG7	*	*	*	*	*	*	*	*	*	C	*	*	*	G	*	*		0.09		
TG8	*	*	*	*	G	*	*	*	*	C	*	*	*	G	*	*		0.184		
TG9	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	T		0.09		
TG10	*	*	*	*	*	*	T	T	*	*	*	*	A	*	*	*			0.5	
TG11	*	*	*	*	*	C	T	T	*	*	*	*	*	*	*	*			0.25	0.333
TG12	*	*	*	*	*	C	T	*	*	*	*	*	A	*	*	*			0.25	0.167
TG13	*	*	*	*	*	C	*	T	*	*	*	*	A	*	*	*				0.5
Nucleotide diversity (p)																	0.006	0.008	0.004	0.004
Number of haplotypes (h)																	5	6	3	3
Haplotype diversity (F_{ST})																	0.752	0.873	0.833	0.733
Number of polymorphic sites (s)																	9	8	3	3

Sampling stations IDs, JOH: Johor, SEL: Selangor, LAN: Langkawi, KED: Kedah, TG1- TG13 represents the observed haplotypes in *Tachypleus gigas*; '*' represents the polymorphic mismatches in mentioned nucleotide position)

Table 4: Pair wise F-statistic (F_{ST}) values of genetic differentiation and migrants per generation (N_em) values of gene flow among populations. F_{ST} values are above the diagonal and N_em values are below the diagonal

Populations (ID)	JOH	SEL	LAN	KED
JOH	-	0.119*	0.534*	0.557*
SEL	3.702	-	0.489*	0.523*
LAN	0.436	0.522	-	0.111^{ns}
KED	0.398	0.456	4.005	-

*indicates significant variation ($p<0.05$) and ns indicates Non significance

this population where as NW Malaysian samples had higher gene flow and lesser haplotype diversity. The overlapping of TG1 and TG3 haplotype in SW Malaysian samples and TG11 and TG12 in haplotype in NW Malaysian samples might be due to the restricted migration of horseshoe crab samples between closest geographical areas (Table 3). This finding was well corresponded with recent study on tri spine horseshoe crab *T. tridentatus* samples from west Japan water samples (Smith *et al*., 2009e) where he observed the dispersal rate of lower haplotype divers samples lead to genetically distinct population. Higher polymorphic sites in SW Malaysian samples compared to NW Malaysian samples indicated comparatively faster genetic mutation (Single Nucleotide Polymorphism) in SW Malaysian *T. gigas* samples.

Population structure and gene flow: The fixation index (F_{ST} value) between Johor vs Selangor samples and Langkawi vs Kedah samples were lower with 0.119 ($p<0.05$) and 0.111 ($p>0.05$), respectively which indicating higher gene flow between these population. This observation was also proved by migratory rate per generation between populations (N_em) which revealed the higher migratory rate between Johor vs Selangor samples (3.702) and Langkawi vs Kedah samples (4.005) (Table 4). This analysis clearly proved the restricted migration of horseshoe crab samples along the west coast of Malaysia. Similar observations were also recorded in other aquatic organisms (Wong *et al*., 2011; Van der Kuyl *et al*., 2005).

CONCLUSION

The results of this study proved the restricted geographical gene flow among the *T. gigas* population along the west coast of peninsular Malaysia. The genetic data presented also proved that the gene flow between NW and SW Malaysian *T. gigas* population is very limited. Additional molecular marker studies need to be addressed on this issue. *T. gigas* population in Malaysian coast is moderately abundant however their population density is constantly being in declining phase along the Malaysian coast resulting from pollution and loss of suitable spawning and feeding ground due to various anthropogenic activities. The genetic structure of local populations provide molecular information that could be for implementing different conservation strategies such as establishing horseshoe crab sanctuaries along the west coast especially in Selangor and Johor area due to higher genetic diversity of *T. gigas*.

ACKNOWLEDGMENT

Author wish to extend his sincere thanks to ministry of Higher Education Malaysia who funded this project under Fundamental Research Grant Scheme (FRGS) (Project Reference Number: 02-10-07-307FR).

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