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Asian Journal of Animal and Veterinary Advances

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Asian Journal of Animal and Veterinary Advances 8 (1): 63-72, 2013
ISSN 1683-9919 / DOI: 10.3923/ajava.2013.63.72

Genetic Diversity of Horseshoe Crab (*Tachypleus gigas*) in Malaysia Revealed using Microsatellite Markers

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ABSTRACT

The genetic structure of horseshoe crab (*Tachypleus gigas*) populations were examined using 18 microsatellite markers. Samples were collected from 2 stations of east coast of Peninsular Malaysia and one station from East Malaysia. All the observed allelic frequency showed no significant variation between sampling stations ($p>0.05$). Mean allelic richness (A_r) was greater in Sarawak population (4.83) followed by Pahang (4.24) and Terengganu (3.57) samples with the overall mean allelic richness of 4.21. Mean inbreeding coefficient (F_{IS}) value was higher in Sarawak population (0.37) followed by Terengganu (0.31) and Pahang population (0.17). The genetic differentiation (F_{ST} value) and genetic distance between geographically closer populations was smaller compared to geographically isolated populations. Due to the dwindling population size of horseshoe crabs in Malaysian coast line, present data provides new information in assisting proper management and conservation of this living fossil.

Key words: Horseshoe crabs, fishery management, microsatellite markers, *Tachypleus gigas*, population genetics

INTRODUCTION

Horseshoe crabs are well known creatures remarkably retaining their genetic makeup virtually unchanged over millions of years (John *et al.*, 2010). Numerous studies were attempted to explore their phylogeny and phylogeography of four extant species using mitochondrial DNA markers (Xia, 2000; Kamaruzzaman *et al.*, 2011) while only few studies were done to investigate their population structure and genetic diversity using microsatellite markers (Li *et al.*, 2009; Faurby *et al.*, 2010). It was noted that all those studies concentrated mostly on specific species such as *Tachypleus tridentatus* and *Limulus polyphemus* while the population genetic studies on *T. gigas* is still limited especially in Malaysia (Rozihan and Ismail, 2011, 2012).

Tachypleus gigas population is established in both Peninsular Malaysia and east Malaysian coast. They migrate to the nesting grounds during full and new moon period to lay their eggs in loosely packed sediments in intertidal area (Akbar John *et al.*, 2012). Field observation revealed the co-occurrence of *T. gigas* and *C. rotundicauda* in some nesting grounds along the east coast

of Peninsular Malaysia. At present, the collection of *T. gigas* samples from the wild habitat and nesting grounds are becoming tedious because of dwindling population size due to habitat degradation besides their commercial fishery and biomedical importance (John *et al.*, 2011). Hence, adequate management of this vulnerable species requires knowledge for their conservation, including information about their ecology and population structure. Here, this study presented the genetic differentiation within and among the *T. gigas* population noted in east coast of Peninsular Malaysia and East Malaysia using microsatellite molecular markers.

MATERIALS AND METHODS

Sample collection and microsatellite genotyping: A total of 31 horseshoe crab samples were collected from 3 states (Terengganu (N 05°41.000' E 102°42.594') = 11 samples; Pahang (N 03° 31.988' E 103°27.534') = 12 samples and Sarawak (N 01°38.944' E 110°28.508') = 8 samples) (Fig. 1). Samples were identified morphologically, sexed and weighed. A sterile scissor was used to excise 2×2 cm of book gill tissue and preserved in the 95% ethanol for further analysis and horseshoe crab samples were released back into the water.

Total DNA was isolated from gill tissue using Promega® Wizard Genomic DNA Purification Kit. 18 microsatellite loci developed for American conspecific species (*Limulus polyphemus*) by King and Eackles (2004) were used in the amplification of scorable bands (Table 1). The amplified products were gel eluded using 5% Polyacrylamide gel. Alleles were sized using BioCapt software using 50 bp allelic ladder for consistent scrolling of alleles.

Data analysis: Van Oosterhout *et al.* (2004) method were adopted to check presence of null alleles and its dominance. GENEPOP 3.3v software was used to check the allelic frequencies and independence of genotypes among loci (Raymond and Rousset, 1995). FSTAT 2.93v was used to calculate genetic diversity parameters such as observed (H_o), expected heterozygosities (H_e) (under Hardy-Weinberg equilibrium) and Ar (allelic diversity) (Goudet, 1995). Pairwise F_{ST} values among sites and their significant variation among the sites (Analysis of Molecular Variance, AMOVA) were calculated using ARLEQUIN 3.1v (Schneider *et al.*, 2000).

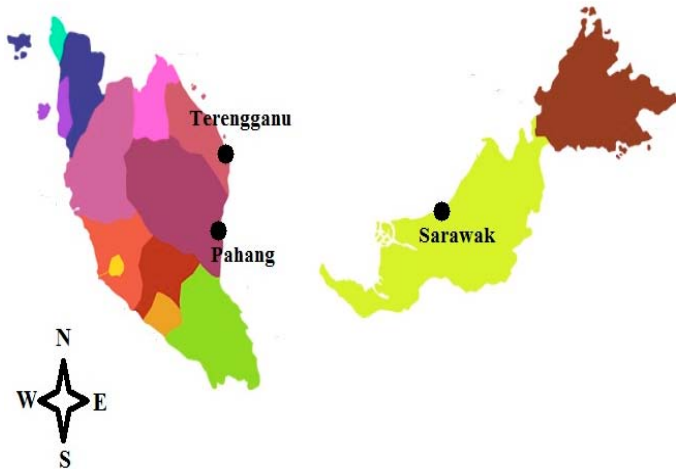

Fig. 1: Location of the sampling are along the East Malaysian coast

Table 1: Primer sequences of 18 polymorphic microsatellite loci that were used to characterize the three *T. gigas* populatious in Malaysia

Locus	Primer sequences 5' to 3'	Size (bp)	GenBank accession No.
LpoA5	F: GTACACAGCAGATAGGCAGATG	177-201	AF534363
	R: ATATAGTGGAGAGACGCGGTAG		
LpoA26	F: GGAAGAAATATTGAAGTTAAAGAAAAG	147-175	AF534364
	R: CAGGCCTAAAGTTGACATACG		
LpoA37	F: CAGCAGATAGTCCAATGTAGCTC	165-181	AF534365
	R: CAAATCAATCATCATAGCAAAATC		
LpoA40	F: ACGATTTGCTTATTTGCACAC	133-175	AF534367
	R: GTAGTGTTATGATGCGTTCAGG		
LpoA42	F: TGTGGGTTTTGTCATTTCATAC	145-173	AF534368
	R: CAACATATTGAGTTGGAGCAAG		
LpoA44	F: AAGGAGCATCCAGTCAAAATC	121-175	AF534369
	R: TATATTTGGTCAGGGTGGAAAG		
LpoA52	F: AGATACACTGATTTTCGTGCAG	155-177	AF534370
	R: ATTATAGCGTGTAGGGCTGAAG		
LpoA56	F: TCTGCAACCAACACAAAACTAC	255-281	AF534371
	R: GTCTTACGATGGGTCTTTTCTC		
LpoA58	F: GGTTCATGTGTTGTTGTTCTTC	86-155	AF534372
	R: GTTTCACGGATGAATTTGTTTC		
LpoA60	F: GCTGAGGTGGGCTAGATAAAAC	106-126	AF534373
	R: CTCCCATATAGCTTTCTCATTTG		
LpoA64	F: ACACGCATATCTATTCTTTTGC	133-153	AF534374
	R: CCTGTGTTTGGTACGTTTAATTC		
LpoA67	F: TGCAATCTAAACTGTCTGAACC	89-155	AF534375
	R: ATTACGAGTTGTTCACCCTGAC		
LpoA68	F: ACCAAAACTCAACAAGGACAAG	147-171	AF534376
	R: ATATTAAGGCAGCATTGACGTG		
LpoA73	F: CACTTAATAGCTGTTGCCAGTC	135-187	AF534377
	R: TCCAACAATCAACCTTATGAATAC		
LpoA309	F: GCAGCAACCAAAGTCAGG	194-218	AF534380
	R: TGGGGCACTTAACTTGTATTG		
LpoA315	F: ACCCAGTTTCATTCTTTTCAC	105-125	AF534381
	R: GCAGTGGCTAACCTGAGTC		
LpoD6	F: AAGTATTGAAGGTGTGGTGGTC	144-282	AF534383
	R: TTCTGTAAGTGTCACGCTATGG		
LpoD60	F: TACATCTACAACCCTACAAGTGC	163-211	AF534384
	R: TGTGCTTAATTACAAGCAAACC		

RESULTS AND DISCUSSION

Allele frequency: A total of 118 alleles were detected at 18 microsatelhte locus noted in horseshoe crab populations. Mean number of allele per locus was 3.7±0.4 in Terengganu, 5±0.9 in Pahang and 5.4±0.8 in Sarawak samples. It is well documented that high mutation rate and presence of more number of allelic variations in microsatellite sequence in a population could be useful in predicting population structure (Goldstein *et al*., 1999). The existence of flanking region in all the microsatellite loci was apparent in this study which eventually reflected polymorphisms in all the locus (Table 2). Similar situation was observed in American conspecific (King and Eackles, 2004).

Mean allele frequency of 18 microsatellite loci in horseshoe crab population showed no significant variation between sampling stations ($p>0.05$) which clearly revealed the existence of

Table 2: No. of allele and its frequency observed at 18 microsatellite loci from three populations of *T. gigas*

		Allele frequency		
Locus name	Allele length	Terengganu	Pahang	Sarawak
LpoA5	100	0.136	0.000	0.000
	125	0.364	0.583	0.563
	150	0.273	0.250	0.250
	175	0.227	0.167	0.125
	200	0.000	0.000	0.063
LpoA26	125	0.455	0.208	0.313
	150	0.318	0.333	0.438
	175	0.182	0.250	0.125
	200	0.000	0.042	0.000
	225	0.045	0.042	0.063
	250	0.000	0.125	0.063
LpoA37	100	0.273	0.000	0.063
	125	0.273	0.583	0.375
	150	0.318	0.208	0.250
	175	0.136	0.125	0.125
	200	0.000	0.083	0.125
	225	0.000	0.000	0.000
	250	0.000	0.000	0.063
LpoA40	100	0.136	0.000	0.000
	125	0.273	0.292	0.188
	150	0.364	0.417	0.625
	175	0.227	0.125	0.125
	200	0.000	0.000	0.000
	225	0.000	0.083	0.063
	250	0.000	0.083	0.000
LpoA42	100	0.000	0.042	0.063
	125	0.227	0.417	0.188
	150	0.636	0.292	0.438
	175	0.136	0.083	0.188
	200	0.000	0.083	0.063
	225	0.000	0.000	0.063
	250	0.000	0.083	0.000
LpoA44	100	0.091	0.000	0.125
	125	0.409	0.458	0.250
	150	0.182	0.292	0.500
	175	0.318	0.083	0.063
	200	0.000	0.083	0.063
	250	0.000	0.083	0.000
LpoA52	100	0.182	0.000	0.063
	125	0.318	0.583	0.500
	150	0.364	0.208	0.125
	175	0.136	0.125	0.125
	200	0.000	0.083	0.125
	225	0.000	0.000	0.000
	250	0.000	0.000	0.063

Table 2: Continue

		Allele frequency		
Locus name	Allele length	Terengganu	Pahang	Sarawak
LpoA56	100	0.000	0.042	0.063
	125	0.227	0.500	0.188
	150	0.636	0.292	0.438
	175	0.136	0.167	0.125
	200	0.000	0.000	0.063
	225	0.000	0.000	0.125
LpoA58	100	0.136	0.000	0.000
	125	0.318	0.292	0.188
	150	0.318	0.417	0.500
	175	0.227	0.125	0.188
	200	0.000	0.000	0.000
	225	0.000	0.083	0.063
	250	0.000	0.083	0.063
LpoA60	100	0.000	0.042	0.063
	125	0.182	0.417	0.188
	150	0.636	0.292	0.438
	175	0.182	0.083	0.063
	200	0.000	0.083	0.125
	225	0.000	0.000	0.125
	250	0.000	0.083	0.000
LpoA64	100	0.136	0.000	0.000
	125	0.273	0.292	0.188
	150	0.364	0.417	0.500
	175	0.227	0.125	0.125
	200	0.000	0.000	0.063
	225	0.000	0.083	0.125
	250	0.000	0.083	0.000
LpoA67	100	0.000	0.042	0.063
	125	0.227	0.375	0.188
	150	0.636	0.333	0.375
	175	0.136	0.083	0.188
	200	0.000	0.083	0.063
	225	0.000	0.000	0.063
	250	0.000	0.083	0.063
LpoA68	100	0.136	0.000	0.125
	125	0.409	0.292	0.250
	150	0.318	0.333	0.375
	175	0.136	0.250	0.063
	200	0.000	0.000	0.125
	225	0.000	0.042	0.000
	250	0.000	0.083	0.063
LpoA73	100	0.182	0.000	0.063
	125	0.409	0.583	0.500
	150	0.273	0.208	0.250
	175	0.136	0.125	0.000
	200	0.000	0.083	0.188
	225	0.000	0.000	0.000

Table 2: Continue

		Allele frequency		
Locus name	Allele length	Terengganu	Pahang	Sarawak
LpoA309	100	0.000	0.042	0.063
	125	0.227	0.417	0.188
	150	0.636	0.292	0.375
	175	0.136	0.083	0.063
	200	0.000	0.083	0.125
	225	0.000	0.000	0.188
	250	0.000	0.083	0.000
LpoA315	100	0.136	0.000	0.000
	125	0.364	0.458	0.500
	150	0.273	0.208	0.188
	175	0.227	0.125	0.188
	200	0.000	0.083	0.063
	250	0.000	0.125	0.063
LpoD6	100	0.136	0.042	0.125
	125	0.409	0.208	0.250
	150	0.318	0.375	0.500
	175	0.136	0.250	0.063
	200	0.000	0.000	0.063
	225	0.000	0.125	0.000
LpoD60	100	0.045	0.000	0.000
	125	0.136	0.292	0.188
	150	0.591	0.417	0.500
	175	0.227	0.125	0.125
	200	0.000	0.000	0.063
	225	0.000	0.083	0.063
	250	0.000	0.083	0.063

more than one population of horseshoe crab in east coast of Malaysia. In general, horseshoe crabs have limited migratory capacity and they migrate to shallow area for reproduction and the juveniles tend to stay at the natal beach for feeding (Sekiguchi, 1988; Chen *et al.*, 2004). This might probably be the reason why they displayed genetic subdivision between populations and isolation by distance. Mean allelic richness (A_r) was greater in Sarawak population (4.83) followed by Pahang samples (4.24) and Terengganu crabs (3.57) with the overall mean allelic richness of 4.21 in east coast Malaysian samples. Mean number of alleles identified at a population is considered to be a good indicator of genetic variability (Leberg, 2002). Compared to the previous report on *L. polyphemus* microsatellite analysis by Orti *et al.* (1997), present study revealed less allelic variation in *T. gigas* population in East coast of Malaysia.

The observed heterozygosity (H_o) ranged from 0.25-0.75 in Sarawak, 0.33-0.75 in Pahang and 0.27-0.64 in Terengganu populations. Expected heterozygosity (H_e) was ranging from 0.62-0.86 in Sarawak population, 0.6-0.81 in Pahang samples and 0.56-0.76 in Terengganu crabs. Mean homo and heterozygosity values in Sarawak population were 0.48 and 0.72 which is slightly greater than Terengganu population (Ho = 0.47; He = 0.69) and lesser than Pahang samples (H_o = 0.59; H_e = 0.72). The fairly low heterozygosity value in Pahang population might be attributed to less number of allelic variations and high level of inbreeding (Aliabadi *et al.*, 2008).

Table 3: Genetic variability at 18 microsatellite loci from three populations of *T. gigas*

Locus	Statious	A_r	H_o	H_e	F_{IS}	P-HWE
LpoA5	Terengganu	3.91	0.455	0.773	0.412	0.132
	Pahang	2.947	0.5	0.598	0.165	0.048*
	Sarawak	3.699	0.625	0.643	0.028	0.687
LpoA26	Terengganu	3.516	0.545	0.695	0.216	0.432
	Pahang	4.865	0.417	0.814	0.488	0.004*
	Sarawak	4.45	0.5	0.75	0.333	0.159
LpoA37	Terengganu	3.916	0.455	0.782	0.419	0.156
	Pahang	3.634	0.667	0.617	-0.08	0.083*
	Sarawak	5.399	0.75	0.813	0.077	0.604
LpoA40	Terengganu	3.91	0.636	0.764	0.167	0.018*
	Pahang	4.411	0.583	0.75	0.222	0.156
	Sarawak	3.693	0.25	0.616	0.594	0.007*
LpoA42	Terengganu	2.913	0.455	0.555	0.18	0.108
	Pahang	4.78	0.667	0.754	0.116	0.458
	Sarawak	5.236	0.375	0.804	0.533	0.004*
LpoA44	Terengganu	3.776	0.455	0.736	0.383	0.146
	Pahang	4.28	0.583	0.72	0.189	0.234
	Sarawak	4.449	0.375	0.732	0.488	0.002*
LpoA52	Terengganu	3.893	0.364	0.768	0.527	0.014*
	Pahang	3.634	0.667	0.617	-0.08	0.083*
	Sarawak	5.35	0.75	0.741	-0.012	0.563
LpoA56	Terengganu	2.913	0.455	0.555	0.18	0.108
	Pahang	3.451	0.583	0.667	0.125	0.076*
	Sarawak	5.393	0.5	0.804	0.378	0.013*
LpoA58	Terengganu	3.911	0.545	0.773	0.294	0.049*
	Pahang	4.411	0.583	0.75	0.222	0.166
	Sarawak	4.486	0.375	0.741	0.494	0.008*
LpoA60	Terengganu	2.943	0.455	0.559	0.187	0.287
	Pahang	4.78	0.667	0.754	0.116	0.458
	Sarawak	5.393	0.5	0.804	0.378	0.028*
LpoA64	Terengganu	3.91	0.636	0.764	0.167	0.018*
	Pahang	4.411	0.583	0.75	0.222	0.158
	Sarawak	4.643	0.375	0.75	0.5	0.006*
LpoA67	Terengganu	2.913	0.455	0.555	0.18	0.108
	Pahang	4.782	0.667	0.761	0.124	0.432
	Sarawak	5.986	0.375	0.857	0.563	0
LpoA68	Terengganu	3.843	0.545	0.736	0.259	0.369
	Pahang	4.251	0.333	0.784	0.575	0.002*
	Sarawak	5.399	0.5	0.83	0.398	0.001*
LpoA73	Terengganu	3.891	0.364	0.759	0.521	0.011*
	Pahang	3.634	0.667	0.617	-0.08	0.083*
	Sarawak	3.742	0.5	0.705	0.291	0.493
LpoA309	Terengganu	2.913	0.455	0.555	0.18	0.108
	Pahang	4.78	0.667	0.754	0.116	0.454
	Sarawak	5.436	0.625	0.83	0.247	0.230*
LpoA315	Terengganu	3.91	0.455	0.773	0.412	0.132
	Pahang	4.525	0.75	0.739	-0.015	0.098*
	Sarawak	4.486	0.5	0.732	0.317	0.049*

Table 3: Continue

Locus	Statious	A_r	H_o	H_e	F_{IS}	P-HWE
LpoD6	Terengganu	3.843	0.545	0.736	0.259	0.369
	Pahang	4.366	0.417	0.784	0.469	0.008*
	Sarawak	4.449	0.375	0.732	0.488	0.002*
LpoD60	Terengganu	3.458	0.273	0.623	0.562	0.003*
	Pahang	4.411	0.583	0.75	0.222	0.154
	Sarawak	5.193	0.375	0.759	0.506	0.004*
Mean	Over all	4.21	0.51	0.72	0.28	
	Terengganu	3.57	0.47	0.69	0.31	
	Pahang	4.24	0.59	0.72	0.17	
	Sarawak	4.83	0.48	0.76	0.37	
Range	Overall	2.91	0.25	0.56	-0.08	
		-5.99	-0.75	-0.86	-0.59	
	Terengganu	2.91	0.27	0.56	0.17	
		-3.92	-0.64	-0.78	-0.56	
	Pahang	2.95	0.33	0.60	-0.08	
		-4.87	-0.75	-0.81	-0.58	
	Sarawak	3.69	0.25	0.62	-0.01	
		-5.99	-0.75	-0.86	-0.59	

Ar: Allele richness, H_o: Observed heterozygosity, H_e: Expected heterozygosity, F_{IS}: Inbreeding coefficient, HWE: Hardy-Weinberg equilibrium (*indicates statistical significance at 95% confidence interval)

Deviation from hardy-Weinberg equilibrium at the locus level had shown significant departure in multiple loci (Table 3). Mean inbreeding coefficient (F_{IS}) value was higher in Sarawak population (0.37) followed by Terengganu (0.31) and Pahang population (0.17).

Genetic variation among sampling stations: The genetic differentiation (F_{ST} value) between geographically closer populations was smaller compared to geographically isolated populations. Lower F_{ST} value was recorded between Pahang and Terengganu samples (0.1441) followed by Terengganu and Sarawak population (0.5496) and Pahang/Sarawak samples (0.8469). However, the values were not statistically significant ($p>0.05$). Pairwise genetic distance value was lowest between Pahang/Sarawak population (-0.0113) followed by Terengganu/Sarawak (0.0047) and Pahang/Terengganu (0.0326) (Table 4). This observation contradicts with previous study on *T. gigas* genetic variation by Rozihan and Ismail (2012). Probable reasons are it might be due to the differential genetic cues in mitochondrial and microsatellite markers besides the number of samples collected from each sites could not reveal distinct genetic distance between geographically separated populations.

Analysis of Molecular Variance (AMOVA) using 18 microsatellite loci showed greater percentage variance among individuals of the same population (28.57%) ($p<0.01$) compared to the individuals of different population (7.47%) (Table 5). AMOVA test revealed the significant difference in sampled population might be due to rapid mutational rate in microsatellite loci (Wirgin *et al*., 2002). Probably the species specific microsatellite markers will reveal more genetic information on the population pattern and inbreeding rate of *T. gigas* population in Malaysian coast line.

Table 4: Estimates of pairwise genetic distance (Nei, 1978; below diagonal) and genetic differentiation F_{ST} (Weir and Cockerham, 1984; upper diagonal) among three populations of *T. gigas*

	Terengganu	Pahang	Sarawak
Terengganu	-	0.1441^{ns}	0.5496^{ns}
Pahang	0.0326	-	0.8469^{ns}
Sarawak	0.0047	-0.0113	-

ns: Not significant

Table 5: Hierarchical analysis of molecular variance (AMOVA) in *T. gigas*

Source of variation	Sum of squares	Variance components	Percentage of variation
Among population	112.346	0.510	07.47
within populations	553.728	1.950	28.57*

*Significant at $p<0.01$

In conclusion, the data presented in this study revealed restricted migratory pattern of horseshoe crab (*T. gigas*) along the east coast of Malaysia. However extensive study need to carried out with more sampling size to conserve their genetic population subdivisions in natural habitat.

ACKNOWLEDGMENT

Author wish to extend his sincere gratitude 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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