Journal of
Fisheries and Aquatic Science

ISSN 1816-4927

Academic
Journals Inc.

www.academicjournals.com

Journal of Fisheries and Aquatic Science 6 (6): 614-623, 2011
ISSN 1816-4927 / DOI: 10.3923/jfas.2011.614.623

Genetic Diversity of Wild and Cultured Populations of *Penaeus monodon* using Microsatellite Markers

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ABSTRACT

Information on the genetic diversity of *Penaeus monodon* throughout its natural range in Malaysia is still limited even though it is a highly exploited species, thus this study was undertaken to genetically characterize the prawn populations. The *P. monodon* samples were randomly collected from Malaysian waters and were characterized using thirty polymorphic primer pairs which showed high level of polymorphism. The total number of alleles per locus ranged from 3 to 36 with allele size ranging from 100 to 275 base pairs. The mean observed heterozygosity (0.5166) was less than the expected (0.5552), highly significant deficiencies in heterozygotes were detected in total inbreeding (F_{IS} = 0.5500) and pair-wise genetic differentiation (F_{ST} = 0.6308) among the populations. Both the (χ^2) chi-square and (G^2) likelihood ratio tests detected significant differences ($p<0.05$) which showed a deviation from the Hardy-Weinberg equilibrium, indicating a probable inbreeding might have occurred in the populations. A Cluster analysis based on genetic distance revealed a fair genetic relationship among all the populations and the pattern was in accordance to the populations' geographical origins. The highest genetic distance (0.7588) was observed between Lawas and Pulau Sayak populations while the lowest genetic distance (0.1191) was recorded between the Endau Rompin and Sedili populations. Various levels of genetic diversity of the *P. monodon* reported in this study indicated their genetic status in Malaysian waters and suitability for breeding and culture purposes. This information provides a basis for improvement through selective breeding and in the design of suitable management guidelines for this genetic material.

Key words: Genetic, variation, *P. monodon*, population structure, conservation, malaysian waters

INTRODUCTION

Penaeus monodon (common names; giant tiger prawn, black tiger prawn) is the most widely cultivated, distributed and marketed prawns in the world. It is widely distributed in the Indo-West-Pacific-ranging from the east coast of Africa, the Arabian Peninsula, South-East Asia, the Sea of Japan and also in Eastern Australia. The estimated aquaculture production of *Penaeus monodon* in Malaysia was 13,503.31 tonnes with an estimated wholesale value of (RM'000) 290,552.71 (DOF, 2008).

The rapid and uncontrolled growth of the shrimp aquaculture industry has drawn public attention to its potential impact on the wild shrimp population and the environment (Benzie, 2000). The international culture of *Penaeus monodon* rapidly grew at the end of the 1980's till the early 2000's with the highest aquaculture production of 700,000 tonnes in 2003. However, the development of the aquaculture industry of the giant tiger prawn especially in the Southeast Asian countries has been reported to have declined with a total of less than 600,000 tonnes aquaculture production in 2007. The contribution has been unofficially reported to have declined particularly in Thailand and Indonesia, because of substitution by *Litopenaeus vannamei* in many cultured farms. Due to this alternative species, the development of *P. monodon* production is expected to slow down in the immediate future, but later it may increase again if the research and development programmes are improve, thus perk up the sustainability of production and low down the operational costs (FAO, 2010).

Information on the genetic structure and diversity of natural populations of *Penaeus monodon* throughout its natural range is still limited (Brooker *et al*., 2000) even though it is a highly exploited species. Thus, knowledge on genetic diversity of natural populations is essential for resource management, breeding and preservation of aquatic diversity (Kocher *et al*., 1998; Liu and Cordes, 2004).

Studies carried out on genetic diversity on marine prawns, *P. monodon, F. merguiensis* using morphological, biochemical and molecular markers are sporadic (Daud, 1995; Pongsomboon *et al*., 2000; You *et al*., 2008). Molecular markers are used as a tool for examining genetic variation and facilitating confirmation of taxonomy as well as for differentiation of two-morphologically-close species and determination of genetic variations (Silverstein *et al*., 2004). Microsatellites are being used as genetic markers in many living organisms such as in poultry animals (Nassiri *et al*., 2007), plant species (Guasmi *et al*., 2008; Ghanbari *et al*., 2009) and aquaculture (Kavan *et al*., 2009; Gharibkhani *et al*., 2009). It has become the markers of choice in genetic mapping and aquaculture research thus, the present study was undertaken to characterize the wild populations of *Penaeus monodon* in Malaysian waters by using microsatellite markers.

MATERIALS AND METHODS

A total of 138 samples of *Penaeus monodon* were collected from six locations in the West and East Malaysia (Fig. 1); Endau Rompin (30), Sedili (20), Gelang Patah (30), (West Malaysia), Lawas (11) and Labuan (17), (East Malaysia), (30). The samples consisted of both male and female individuals. The cultured prawns of the Pulau Sayak population originated from Mozambique, Africa. These specific pathogen free prawns were imported in 2006 by the Department of Fisheries in collaboration with a private company for the development and production of SPF prawn broodstocks programme in Malaysia.

DNA extraction: DNA was extracted from muscle tissue (20 g wet weight) by using Promega® Wizard Genomic DNA Purification Kit based on modified protocol of Wanna *et al*. (2004). DNA quality was checked on 0.8% agarose gel. The quantity of the DNA was further quantified by using a spectrophotometer (examined at absorbance of A_{280}). The DNA quality that fell within the range of 1.8-2.0 was analyzed for Polymerase Chain Reaction (PCR) amplification.

Primer screening: A total of 30 primer pairs (10 from *Fenneropenaeus merguiensis* and 20 from *Penaeus monodon* microsatellite primers (Table 1, 2) obtained from Genbank, Biobasic Canada were used to cross amplify the samples. The PCR amplification conditions for each primer pair were

Fig. 1: Map of Malaysia with alphabets (A-F) indicating sampling sites of *Penaeus monodon*. A: Endau Rompin, B: Sedili, C: Labuan, D: Lawas E: Gelang Patah F: Pulau Sayak

Table 1: List of DNA microsatellites and cross amplified primer pairs design for *Fenneropenaeus merguiensis*

Locus ID	Gene bank accession No.	Primer sequence (5'-3')	Size (bp)	Amplification
GT242	DQ388003	F: CACACGAGTTGATTGTCTGCT	316	Polymorphic
		R: CACGTTGTGTGTGCAGTGAG		
GT273	DQ388004	F: CTTGCATAACCGGGATTTGT	249	Polymorphic
		R: CAACCCAAACACGCATACAC		
TAA5	DQ388005	F: TGATTTACATGGGTGGCAGA	311	Polymorphic
		R: AATGATGGCATAATGATAACACAA		
TAA15	DQ388007	F: CAAGGACCCACCAAACACAAT	299	Polymorphic
		R: AATTCGAGACATCCCTTTGC		
TAA16	DQ388008	F: GGCTTTTTCGTGACATTGGT	312	Polymorphic
		R: CACACACTGCACACATGGAA		
TAA19	DQ388009	F: AAGGACAGGGAGGAGAGAGG	193	Polymorphic
		R: ACAGGCTGACCAAGGAACAC		
TAA20	DQ388010	F: TGTCGCTAAAAATCCAAAAGG	158	Polymorphic
		R: TGCTGTCCATAATCTGCATTG		
TAA25	DQ388011	F: GCTGTCATCATCATTAACCTCCT	252	Polymorphic
		R: CGCGTTTATGCCGATATTTT		
TAA28	DQ388012	F: AATGAAGAGCAAGGCTGGTC	181	Polymorphic
		R: CATCATCATCATCATCATTCTGT		
TAA30	DQ388013	F: CGGAGGTCTGCACTCTCTG	221	Polymorphic
		R: CCCCGTATAAAGACGGGATT		

optimized according to the followings: 1 cycle for 3 min at 94°C; 34 additional cycles consisting of 1 min of denaturation at 94°C, 1 min at specific annealing temperatures (50, 52 and 54°C) and 1 min extension at 72°C.

Out of the 30 primer pairs tested, twenty two *F. merguiensis* and *P. monodon* primers were able to cross amplify the six populations namely TAA5, TAA15, TAA16, TAA19, TAA20, TAA25, TAA28, TAA30, GT242, GT273, AY856, AY857, AY858, AY859, AY860, AY861, AY862, AY864, AY866, AY868, AY869 and AY875. The PCR products were resolved using 4% metaphor agarose gel electrophoresis. Allele sizes were determined relative to the 25 base pair molecular weight Promega ladder. The gel was stained with gelred (10 μL 1000 mL^{-1} ddH_2O) for 30 min and visualized under a UV transilluminator and banding patterns were scored.

Table 2: List of DNA microsatellites and primer pairs used in *Penaeus monodon*

Gene bank accession No.	Primer sequence (5'-3')	Size (bp)	Amplification
AY500855	F: GTGTTATTTTCCACGGGTGC	245-325	No amplification
	R: GCTGCAGGAAGTGTGTGCG		
AY500856	F: AACTGCCTACAGTGTGTGCG	250-340	Polymorphic
	R: GAATGGAGCCTGTTGGTTTG		
AY500857	F: TTCACGACCCAGTATGTCCA	232-406	Polymorphic
	R: CAGGTCGCAGGCTCATATTT		
AY500858	F: GTTGCGACGGGTTGATTC	261-433	Polymorphic
	R: TTTATGGCTATGGCTGACAC		
AY500859	F: TAATTTCTCTGCAACGTCCT	268-330	Polymorphic
	R: CTGCTCATTATCCAGTCCAT		
AY500860	F: GATATTTCAAGGAATGCTCG	143-229	Polymorphic
	R: TAATTCGTGCCTTACCTCAT		
AY500861	F: GAACGTCGGGGGATTTACTT	371-441	Polymorphic
	R: ACTATCACACCGAGGCTTGG		
AY500862	F: TAATGGGCGTAAGTCTTCGG	193-303	Polymorphic
	R: TGAAAGGAGTCGGGATATGC		
AY500863	F: TCTTGGTCGGAATGGGTAAG	184-316	No amplification
	R: TTCTGAGAAGGCACACATGC		
AY500864	F: TTTGGACTTCACATCGGTG	234-312	Polymorphic
	R: CGGCTGAACAGGTCTGAAAT		
AY500865	F: CTTCCAAGCGACAGACGAGT	177-255	No amplification
	R: CGAATGCACTGCCTGTATGT		
AY500866	F: CTTTTTGAAATCGCCCTGTT	243-377	Polymorphic
	R: CATTCATCCCGCTCTTCTGT		
AY500868	F: CATTCACACACGTTCACCTG	227-335	Polymorphic
	R: CCCAGTTCACGTATCGTGTG		
AY500869	F: GCTTGCGTGTGTGCATACTT	275-431	Polymorphic
	R: GTTCCCCTCGTGTTTACGAA		
AY500875	F: AAAAGCCAGAGGAAACGTG	172-246	Polymorphic
	R: ACAGTGCACGTACCCACAAA		
AY188972	F: GGTCTTGCTTCCCTAACC	379	No amplification
	R: ATTCTCTTCATCTTTCCGAGTCTGC		
AY188973	F: TGTTCCCCTCACTGTGCCTAC	310	No amplification
	R: GGGAAGCGCCTGTAGAGTAGAG		
AY188974	F: AGGACCTGCATTTGTGTCG	217	No amplification
	R: ATGGCGAGACAAGGTTCG		
AY188975	F: TGCATACCTAACGTACCTACACA	665	No amplification
	R: AAGCTGAATGCAGGTCGAGT		
AY188977	F: CGGATGAACTGTCAGTGGAC	415	No amplification
	R: TTAGCCACGGAAGAAGCACT		

Statistical analyses: Wright's test (Wright, 1984) was used to determine departure from Hardy-Weinberg Equilibrium (HWE) (Hardy, 1908) using PopGene computer programme (version 1.32; Yeh and Boyle, 1997). Allelic frequencies were estimated from genotypes that assume co-dominance. The mean number of alleles per locus and the mean heterozygosity were used to measure the genetic variability of all the populations. F-statistics were used to measure the deficiency or excess of heterozygosity as well as population differentiation and chi-square goodness of fit tests and the G log-likelihood ratio tests were used to determine if the observed genotypic

numbers are consistent with the Hardy-Weinberg expectations for each population. The genetic distance was calculated using Nei (1978).

RESULTS AND DISCUSSION

Thirty microsatellite markers from *P. monodon* and *F. merguiensis* were tested for amplification on genomic DNA of *P. monodon*. Although amplification failures were observed 57% of the microsatellites could be successfully amplified by PCR of which twenty-two primers were polymorphic (Fig. 2).

Genetic diversity: A total number of alleles per locus ranged from 3 to 36 with the allele size ranging from 100 to 275 base pairs. Overall, the mean observed heterozygosity (0.5166) was less than the expected heterozygosity (0.5552) for all the populations (Table 3), indicating deficiency in heterozygotes. This might be due to null alleles, scoring error, inbreeding, or the Wahlund effect (Wahlund, 1928). Null alleles are the result of divergence in the sequences of the flanking regions of the microsatellite marker (Smulders *et al*., 1997; Lavi *et al*., 1994). Inbreeding may also contributed to the deficiency in heterozygosity, although it is a highly plausible point since the *Penaeus monodon* stocks were obtained from the wild which were supposed to be out-bred unless there was a form of bottlenecking in the prawn populations. A more probable reason for the deficiency in heterozygosity is the mutation at the flanking regions of the primer binding sites. The majority of the observed heterozygosity in this study was lower than the expected heterozygosity for each population. Effective number of alleles is also a good measure of the genetic variation, especially in conservation genetic study. Sometimes its effect on populations put more emphasis, but effective number of alleles is easily affected by sample size (Maudetr *et al*., 2002).

Genetic differentiation and relationships among populations: The mean estimate F_{ST} obtained was 0.6308 while the mean estimate gene flow (N_m) value was 0.1463. Primer AY866 showed the highest N_m with value of 0.3336 while primer TAA20 was 0.0133 (Table 4). The overall F_{IT} and F_{IS} values were low but highly significant ($p<0.05$) throughout the populations, suggesting inbreeding within population might have occurred.

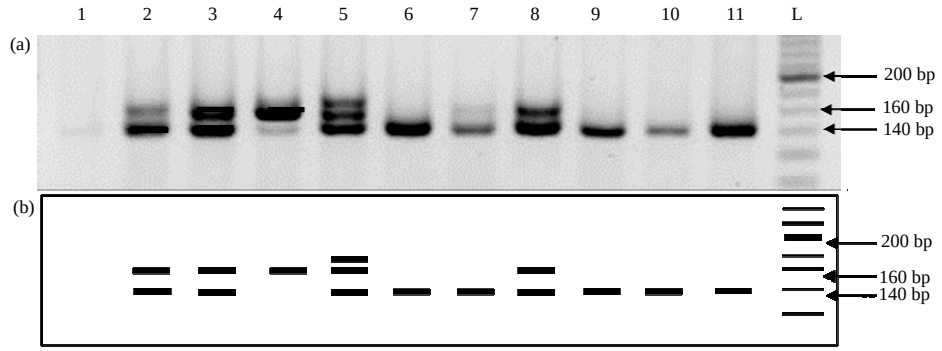

Fig. 2(a-b): (a) Microsatellite banding profile of *P.monodon* samples from Endau Rompin using primer pair TAA19. (b): A diagrammatic representation of the microsatellite bands in (a). Lane L: Cambrex 20bp extended ladder. Lane 1-11: Individuals from Endau Rompin

Table 3: The heterozygosity of all six populations of *Penaeus monodon* for all twenty-two microsatellite loci

Locus	Observed heterozygosity	Expected heterozygosity*	Nei**	Average heterozygosity
AY-856	0.8209	0.6933	0.6881	0.4040
AY-857	0.5333	0.6137	0.6086	0.3296
AY-858	0.5800	0.4269	0.4226	0.0983
AY-859	0.6429	0.6514	0.6456	0.3226
AY-860	0.5873	0.7180	0.7123	0.3894
AY-861	0.5616	0.7028	0.6980	0.3495
AY-862	0.7447	0.5822	0.5791	0.3700
AY-864	0.5889	0.4657	0.4631	0.3189
AY-866	0.7753	0.6808	0.6770	0.4543
AY-868	0.5644	0.5393	0.5367	0.3833
AY-869	0.9000	0.5987	0.5837	0.0973
AY-875	0.7465	0.6299	0.6255	0.3758
TAA-16	0.0278	0.5707	0.5667	0.0911
TAA-19	0.3016	0.5256	0.5214	0.2456
TAA-20	0.0714	0.3249	0.3226	0.0376
TAA-25	0.6429	0.7020	0.6978	0.3143
TAA-30	0.6613	0.7256	0.7227	0.3427
GT-242	0.3190	0.6963	0.6933	0.3536
TAA-5	0.0000	0.0000	0.0000	0.0000
TAA-15	0.5000	0.3782	0.3750	0.0983
TAA-28	0.0000	0.0000	0.0000	0.0000
GT-273	0.2791	0.4326	0.4275	0.0991
Mean	0.5166	0.5552	0.5508	0.2607
Std. Deviation	0.2578	0.1744	0.1733	0.1423

Sixteen loci significantly contributed to the within-population deficit in heterozygosity (F_{IS}), with the mean value of -0.2189. The mean F-statistics implied that 37% of the total genetic variation was from genetic differentiation within each populations and 63% of the genetic variation existed among populations (Table 4).

A further breakdown of the within-population inbreeding estimate for the six populations of *P. monodon* showed a significant deficit of heterozygotes ranging from 0.0717 to 0.9000 in West and East Malaysia. The F_{IS} value for the most inbred locus (TAA16) differed significantly from zero ($p<0.05$) in all the populations which indicate that there is no gene which was fixed within the populations relative to the total population. This showed that the populations despite the presence of homozygosity level are still high in gene variations (Table 4).

Chi-square (χ^2) tests and likelihood ratio (G^2) tests showed that most of the population significantly deviated ($p<0.05$) from Hardy-Weinberg equilibrium for all loci, as the *P. monodon* are marine-estuarine species there are no specific barrier to prevent migrations and genetic flow. The N_m also showed that there is still some form of interaction via migration and mating between the populations despite the geographical distance (Table 4). This situation is similar to the discovery made by Hualkasin (2004) on *Fenneropenaeus merguiensis* in the Thai waters using COI and also to the *F. merguiensis* in Malaysian waters (Daud, 1995) and the lacking of diversity was also stated in a microsatellite study conducted on *Penaeus monodon* from Phillipines (Xu *et al*., 2001). These reviews supported the results of number of alleles and heterozygosity values from obtained this study.

Table 4: The value of F-statistics for all the loci across the six populations of *Penaeus monodon* based on data generated utilizing twenty-two microsatellite loci

Locus	Fis	Fit	Fst	Nm*
AY-856	-0.2885	0.3882	0.5252	0.2260
AY-857	0.0088	0.5938	0.5902	0.1736
AY-858	-0.6949	0.8211	0.8945	0.0295
AY-859	-0.2649	0.5091	0.6119	0.1586
AY-860	-0.1574	0.4975	0.5659	0.1918
AY-861	-0.0203	0.5125	0.5222	0.2287
AY-862	-0.5424	0.2012	0.4821	0.2686
AY-864	-0.3610	0.2813	0.4720	0.2797
AY-866	-0.4305	0.1823	0.4284	0.3336
AY-868	-0.1936	0.3386	0.4459	0.3107
AY-869	-0.5418	0.8482	0.9016	0.0273
AY-875	-0.2667	0.4299	0.5499	0.2046
TAA-16	0.8171	0.9803	0.8921	0.0302
TAA-19	0.1360	0.7597	0.7219	0.0963
TAA-20	-0.0790	0.9454	0.9494	0.0133
TAA-25	-0.2766	0.5225	0.6260	0.1494
TAA-30	-0.4162	0.3903	0.5695	0.1890
GT-242	0.2478	0.6635	0.5526	0.2024
TAA-5	****	1.0000	1.0000	0.0000
TAA-15	-0.6949	0.8209	0.8943	0.0295
TAA-28	****	****	0.0000	****
GT-273	-0.5522	0.8388	0.8962	0.0290
Mean	-0.2189	0.5500	0.6308	0.1463

* Nm = Gene flow estimated from Fst = 0.25 (1 - Fst)/Fst. **** = null allele, (-) = negative value indicating inbreeding in individuals

The individuals from the populations may have undergone a bottleneck effects which cause some of the populations of the *P. monodon* to have more homozygote individuals. Scoring errors could have contributed to the deviation in Hardy-Weinberg equilibrium and the effects of inbreeding may also contribute to the deficiency in heterozygosity. Such deviations from Hardy-Weinberg equilibrium were also observed in various *P. monodon* populations from various regions in Asia including Thailand (Supungul *et al*., 2000) and the Indo-Pacific region (You *et al*., 2008).

Considerable genetic distances were observed between all possible pairs of the *P. monodon* populations. The genetic distance values ranged from 0.1191 to 0.7588. The highest genetic distance was observed between Lawas and Pulau Sayak populations, followed by Labuan and Gelang Patah with a value of 0.6124. As the locality of Pulau Sayak was furthest from Lawas, it was not surprising that the genetic distance between the two populations was the furthest among the populations studied. The Endau Rompin and Sedili populations showed the least genetic distance with a value of 0.1191 (Table 5). This is in agreement with the results obtained for the *P. monodon* where significant distances were observed between the Gulf of Thailand (Tassanakajon *et al*., 1998) and in the Australian wild populations (Li *et al*., 2007).

The UPGMA dendrogram constructed based on the genetic distances generated from twenty two microsatellite loci showed three separate clusters (Fig. 3). The East Malaysian populations clustered further into two groups; one representing eastern part (Lawas) and the other representing the western part (Labuan). The Endau Rompin and Sedili populations were clustered in the same group from the West Malaysia and the cultured prawns from Gelang Patah and Pulau

Table 5: Genetic distance (below diagonal) and identity (above diagonal) based on data generated utilizing twenty two microsatellite loci

POP ID	Endau	Sedili	Lawas	Glg patah	Pulau sayak	Labuan
Endau	****					
Sedili	0.1191	****				
Lawas	0.4581	0.5456	****			
Glg Patah	0.5695	0.4904	0.3931	****		
Pulau Sayak	0.5948	0.3971	0.7588	0.4331	****	
Labuan	0.3424	0.328	0.4593	0.6124	0.6047	****

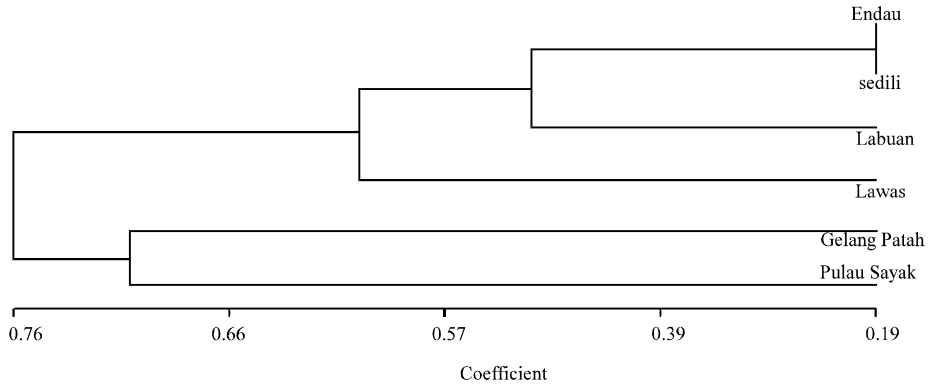

Fig. 3: Dendrogram constructed based on genetic distance values clustered by UPGMA for six populations of *Penaeus monodon* utilizing data from twenty two microsatellite loci

Sayak were in another cluster by itself. Similar population clustering according to their geographical locations was previously observed in the analysis of the black tiger prawns in Indonesia (Sugama *et al*., 2002) and in the Thailand which revealed the occurrence of various patterns of geographic variation in wild stocks (Benzie, 2000).

CONCLUSIONS

In conclusion, most of the populations still exhibited a moderate to high genetic diversities which is important for future breeding development. The populations from the eastern part of Malaysia showed higher genetic diversity compared to the western part. However, there are populations from the western part of Malaysia which are likely becoming genetically homogenous unless effective and appropriate breeding management practices are implemented.

ACKNOWLEDGMENT

The authors greatly acknowledged the Department of Fisheries for the research grant, the staffs of Department of Aquaculture and Department of Animal Science, Faculty of Agriculture, UPM and staffs of Brackishwater Aquaculture Research Center Gelang Patah, Johor for their valuable suggestion, co-operation, the technical support and laboratory facilities provided.

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