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Full Length Research Paper

Genetic diversity of intensive cultured and wild tiger shrimp *Penaeus monodon (Fabricius*) in Malaysia using microsatellite markers

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Inbreeding as negative factor perhaps promote a significant increase of the genetic similarity of the captive populations, consequently leading to greater disease susceptibility and impairment of both the growth and final size of the shrimps. Therefore evaluating genetic diversity and inbreeding required for improvement of brood stock management will assist shrimp breeders to minimize or avoid inbreeding coefficient. The objective of this study was to assess the genetic diversity of intensive cultured and wild tiger shrimp *Penaeus monodon* (Fabricius) in Malaysia using six microsatellite markers (CSCUPmo1,CSCUPmo2,CSCUPmo3,CSCUPmo4,CSCUPmo6 and CSCUPmo7). The mean numbers of allele, observed heterozygosis, and polymorphism information content (PIC) index were calculated. The observed allele's sizes at the investigated loci were similar to those of previous reports. The number of alleles yielded by all microsatellites ranged from 2 to 6 alleles. The highest and the lowest effective number of alleles were found in CSCUPmo1 (4.16) and CSCUPmo7 (1.3), respectively. The most frequent alleles (MFA) for CSCUPmo1, CSCUPmo2, CSCUPmo3, CSCUPmo4, CSCUPmo6 and CSCUPmo7 bp were 259, 165, 181, 242, 206 and 218 bp, respectively. Brood stock showed lower genetic diversity value than wild population. However, with an observed heterozygosity (H_{obs}) below expectations it would be necessary to introduce cross breeding among hatcheries to reduce the risk of inbreeding depression. Microsatellite markers analysis was able to characterize the genetic divergence between the brood stocks and wild population and could be helpful tools for defining better management strategies of these *P. monodon* (Fabricius) in Malaysia.

Keywords: Inbreeding, *Penaeus monodon* (Fabricius), Malaysia, microsatellite markers.

INTRODUCTION

Aquaculture industry has been reported as an industry which has the great potential of becoming a good platform for food security and self sufficiency of any countries. One of the major breaches in an aquaculture is penaeid shrimp farming which is rapidly increasing worldwide (Rosenberry, 1999). *P. monodon* is a marine species that is widely reared for food (FAO, 2010). The natural centers of distribution for this species are Indo-West-Pacific, ranging from the eastern coast of Africa, the Arabian Peninsula, as far as South-east Asia, and the Sea of Japan. They can also be found in eastern Australia, and a small number have colonized the Mediterranean Sea via the Suez Canal. The tiger shrimp, *P. monodon* (Fabricius) could be one of the largest penaeid shrimps in the world, reaching 270 mm in body length and it is of considerable commercial importance in

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Abbreviations: **dNTP**, Dinucleotide triphosphate; H_O, observed heterozygosity; **PCR**, polymerase chain reaction; **MFA**, most frequent alleles; **SSRs**, simple sequence repeats; **PIC**, polymorphism information content.

international markets (Motoh, 1985). The artificial propagation of *P. monodon* was successfully done for the first time in 1968 and the technique has been widely used specially in South East Asian countries (Liao et al., 1969). The shrimp industry in Malaysia has developed rapidly since the early 1980s after the so-called successes experienced in neighboring Thailand, Indonesia and Philippines. Malaysia, however, is not one of the major producers of cultured marine prawn in the world (Azliya, 2009). The Malaysian program on tiger shrimp started in 2001 and this program produced 3 generations of *P. monodon* brood stock in 2006 (Subramaniam et al., 2006). The program in Malaysia started with wild collected shrimp, which passed through a primary quarantine and were screened for multiple viral pathogens. The culture of this species has shown rapid development during the last decade (Suraswadi, 1995). However, the farming of such species relies entirely on wild females for the supply of juveniles. This may result in over-exploitation of the female brood stock in natural populations. Therefore one of the critical steps of fishery management is to understand the population structure of any exploited species.

The recognition of genetic diversity of cultured and wild populations within a species is vital for their raring management. In long term, the most important goal of brood stock management is to control inbreeding and decreasing of growth traits (Allendorf and Ryman, 1987; Ryman, 1991). In artificial pools and cultured populations of brood stock management of shrimp mating among relatives is known as inbreeding, and is a common phenomenon. Even though inbreeding within a cultured population is not itself bad, its accumulation over several generations increases the probability of expression of recessive detrimental alleles when paired in the homozygous state (Tave, 1993). Consequently, such kind of event in inbreeding depression occur leading to a decrease in performance for traits such as growth rate, survival and viral susceptibility. In marine shrimp, some of these problems have already been documented (Bierne et al., 2000; Keys et al., 2004; Moss et al., 2007; Goyard et al., 2008). Evaluating genetic diversity and inbreeding could enhance brood stock management in assisting shrimp breeders to minimize or avoid inbreeding, especially within genetic improvement programs in which inbreeding can accumulate in few generations. Thus, it will be essential to search for proper tools or indicators which can show genetic diversity and information in DNA level to avoid inbreeding in brood stocks of local intensive shrimp farming in Malaysia. Microsatellites, or simple sequence repeats (SSRs) were discovered in 1981. They are tandem repeated motifs of one to six nucleotides found in all prokaryotic and eukaryotic genomes (Goldstein et al., 1995). Dinucleotide repeats dominate, followed by mono and tetra nucleotide repeats and trinucleotide repeats are least dominant.

Repeats of five (penta-) or six (hexa-) nucleotides can also be found. Generally, among dinucleotides, $(CA)_n$ repeats are most frequent, followed by $(AT)_n$, $(GA)_n$ and $(GC)_n$; the last type of repeat being rare (Ellergan, 2004). In the aquaculture industry, microsatellites represent the markers of choice for many applications, for instance genetic monitoring of farmed stocks in view of breeding programs. They allow the analysis of genetic variability and pedigree structure, to design beneficial crosses, select genetically improved stocks, minimize inbreeding and increase selection response (Chistiakov, 2006). Most authors had referred the dinucleotide repeats as the most useful ones for genetic analysis and lately some of them have been considering tri and tetra-nucleotides for the same purpose (Tasanakajon et al., 2000). The objective of this study was identification of polymorphism and genetic diversity of intensive cultured and wild tiger shrimp *P. monodon* (Fabricius) in Malaysia using microsatellite markers.

MATERIALS AND METHODS

Samples and DNA extraction

A total of 50 individuals of wild and cultured *P. monodon* in both sex (25, 25) were randomly selected and used in investigation of the polymorphism. The cultured individuals were taken from Penang pools and about wild individuals Sabah regions were placed for sampling. All the samples were immediately labeled and were packaged using aluminum foil and were kept inside ice flask till it reached the laboratory. Genomic DNA was extracted from the pleopod of a mature shrimp using Commercial Kit (Qiagen, USA) according to the manufacturer's instruction. Purity of the entire extracted DNA was assessed by calculating the OD_{260}/OD_{280}nm ratio determined with Spectrophotometer method. Figure 1 shows the result of DNA extraction. The sequences of the forward and reverse primers for the amplification of the microsatellite are given in Table 1.

PCR assay

PCR was carried out in 25 µl volumes comprising of 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.01 mM of each primer (Fermentas), 50 ng of genomic DNA and 0.2 U Taq DNA polymerase. The PCR protocol comprised of an initial denaturation for 3 min at 94°C, followed by 34 cycles of denaturation for 45 s at 94°C, annealing for 1 min at temperatures specific for the marker (Table 1) and extension at 72°C for 45 min and a final extension at 72°C for 10 min. The products were separated by electrophoresis using a metaphor gel (4%) and the gels were stained with 0.5 mg/l ethidium bromide for 20 min and the banding patterns were visualized under UV.

Statistical analysis

After PCR, the result of reading gel was put in excel sheet and an allele binning used for minimums genotyping error originated from conventional gel. Actual genotype size was considered in excel and then converter software was used to convert data to POPGENE format. The data was analyzed with POPGENE 3.1 (Yeh et al., 1999).

RESULTS

All investigated loci amplified were successful and

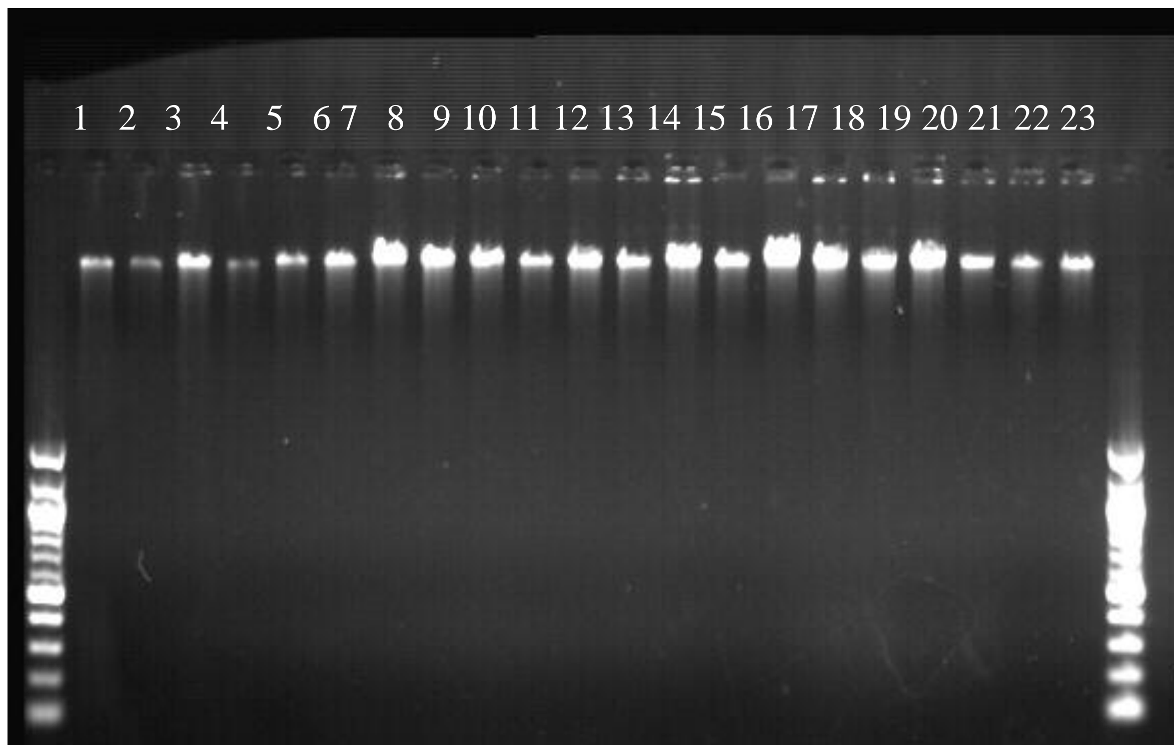


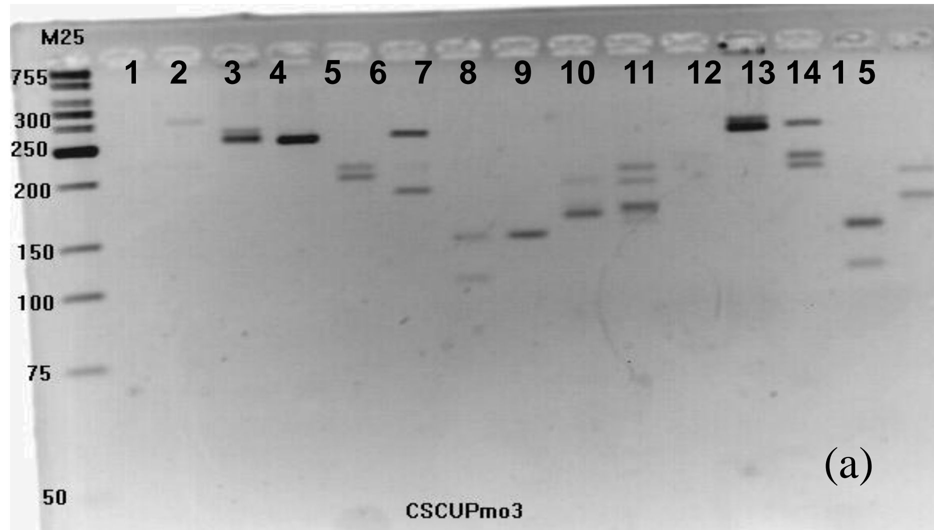
Figure 1. DNA extraction of wild and cultured *P. monodon* using commercial kit.

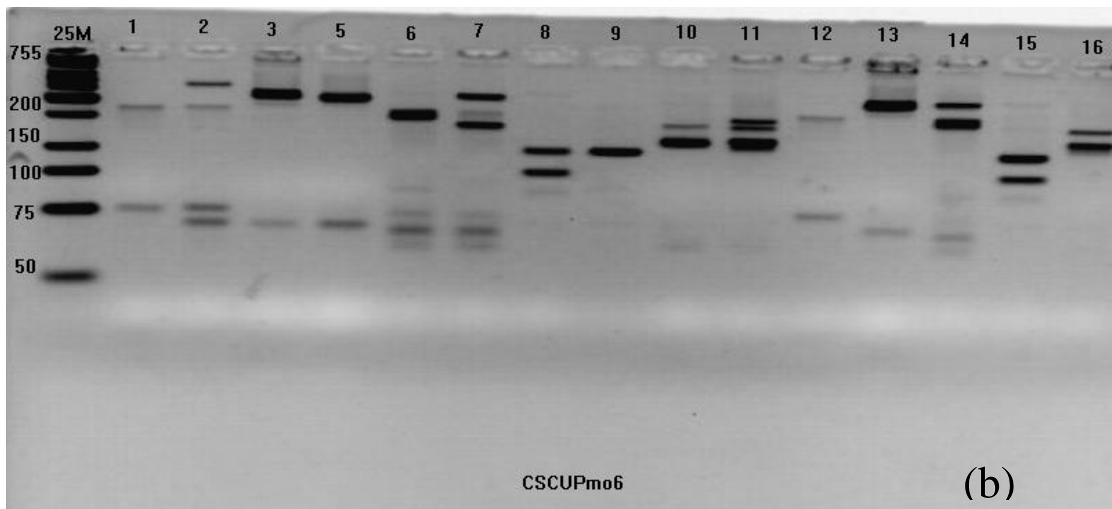
Table 1. Investigated microsatellite loci, motif and primer sequence and annealing temperature for this study.

Locus	Motif	Primer sequence	Reported allele size	Annealing	Reference
CSCUPmo1	(GAA)43	F = ATGATGGCTTTGGTAAATGC R = CGTACTTCCTCTTCATAGGTATC	224-326	56	Pongsomboon et al.(2000)
CSCUPmo2	(ATCT)12(TA)10(TAGA)3	F = CCAAGATGTCCCCAAGGC R = CTGCAATAGGAAAGATCAGAC	137-217	56	Pongsomboon et al.*(*2000)
CSCUPmo3	(ATCT)12(AT)9T(GT)9	F = TGCGTGATTCCGTGCATG R = AGACCTCCGCATACATAC	135-223	56	Pongsomboon et al.(2000)
CSCUPmo4	(CT)10TG(CT)17(ATCT)10	F = TTTCTTTCTTCTCGTGATCCC R = GACGGCATGAGGAATAGAGG	206-256	52	Pongsomboon et al.(2000)
CSCUPmo6	(GATA)6(GA)16	F = TAGTGTTACTCAGGTGCAGC R = GCGTGTATTTGTGATTTCAC	166-242	56	Pongsomboon et al.(2000)
CSCUPmo7	(CT)15(ATCT)9	F = ACGAATGAATGCGGTGGTGC R = TCGGTGCCCAGTTGTATGAGAG	172-234	56	Pongsomboon et al.*(*2000)

F, Forward primer; R, reversed primer.

showed PCR product on gel. Figure 2 shows the pattern of amplification in some of the investigated locus in this study. The number of alleles yielded by all microsatellites ranged from two to six alleles. The highest and the lowest effective number of alleles were found in CSCUPmo1 (4.16) and CSCUPmo7 (1.3), respectively. The MFA for CSCUPmo1, CSCUPmo2, CSCUPmo3, CSCUPmo4, CSCUPmo6 and CSCUPmo7 bp were 259, 165, 181, 242, 206 and 218 bp, respectively. Brood stock showed lower genetic diversity value than wild population. However, with an observed heterozygosity (H_{obs}) below expectations, it would be necessary to introduce cross breeding among hatcheries to reduce the risk of inbreeding depression. Table 2 shows frequencies of each allele and most frequent allele per locus. Number of observed alleles, effective alleles, allele frequencies and length of alleles in base pairs for each locus are given in Tables 3 and 4 which shows the observed and expected





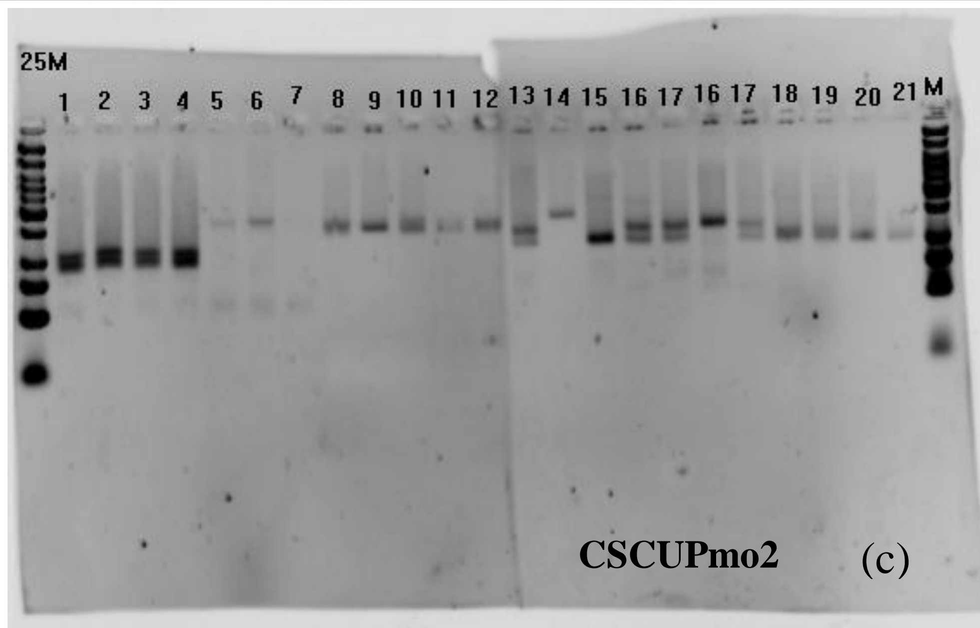


Figure 2. Electrophoresis and genotype size of CSCUPmo3 (a), CSCUPmo6 (b) and CSCUPmo2 (c) of wild and cultured *P. monodon* in this study. 25 ladder used size marker.

Table 2. Summary of PCR product size, frequencies of each alleles per loci and most frequent allele per loci and number of observed allele in investigated of microsatellite in this study.

Locus	CSCUPmo1				CSCUPmo2				CSCUPmo3			
	Wild		Culture		Wild		Culture		Wild		Culture	
	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq
1	233	0.0667			137	0.1333	137	0.1000	156	0.1667	156	0.1667
2	245	0.0333	245	0.0333	157	0.1000	157	0.7000	178	0.3000	178	0.3000
3	251	0.2333	251	0.2333	165	0.5667	165	0.2000	181	0.0333	181	0.0333
4	259	0.3667	259	0.6000	187	0.2000			184	0.3333	184	0.4667
5	269	0.1333	269	0.1333					186	0.0333	186	0.0333
6	317	0.1667							240	0.1333		
Locus	CSCUPmo4				CSCUPmo6				CSCUPmo7			
	Wild		Culture		Wild		Culture		Wild		Culture	
	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq
1	238	0.0667	238	0.1000	166	0.1000	166	0.1000	182	0.1000		
2	240	0.1000	240	0.1333	206	0.5667	206	0.7000	184	0.0667		
3	242	0.7000	242	0.7667	208	0.1000	208	0.2000	212	0.1333	212	0.1333
4	245	0.1333			212	0.2000			218	0.6000	218	0.8667
5					242	0.0333			220	0.1000		

Freq, Frequency; bold number indicated most frequent allele (MFA).

Table 3. Observed number of alleles, effective number of alleles and Shannon's Information index per each microsatellite in this study.

Locus	Sample size	Observed number of allele		Effective number of allele	Shannon index
CSCUPmo1	50	Wild(25)	6	4.1667	1.5686
		Culture(25)	4	2.3077	1.0281
CSCUPmo2	50	Wild(25)	4	2.5714	1.1427
		Culture(25)	3	1.8519	0.8018
CSCUPmo3	50	Wild(25)	6	4.0179	1.5214
		Culture(25)	5	2.9605	1.2422
CSCUPmo4	50	Wild(25)	4	1.9149	0.9291
		Culture(25)	3	1.6245	0.7026
CSCUPmo6	50	Wild(25)	5	2.6163	1.2176
		Culture(25)	3	1.8519	0.8018
CSCUPmo7	50	Wild(25)	5	2.4862	1.2162
		Culture(25)	2	1.3006	0.3927
Mean ±St.dev	Wild (25)	5.000±0.89		2.9622±0.9122	1.2659±0.2410
Mean ±St.dev	Culture (25)	3.333±1.0328		1.9828±0.5813	0.8282±0.2892

hemozygosity and heterozygosity, PIC and average heterozygosity per each microsatellite in this study. Figures 3 and 4 show the observed number of alleles and hetrozygosity per each microsatellite in this study. The highest and lowest F_{st} values were found in CSCUPmo7 (0.0545) and CSCUPmo4 (0.0051), respectively.

DISCUSSION

Cultured shrimp are often intentionally inbred in an effort to enhance overall size and growth potential. A strict correlation between loss in genetic variation and shrimp production performance is well documented (Dumas and Ramos, 1999; Ibarra, 1999; De Beausset et al., 2001), thereby reducing their genetic diversity, that is, adaptive potential, relative to their wild counterparts (Sbordoni et al., 1986; Harris et al., 1990; Sunden and Davis, 1991; Wolfus et al., 1997; Xu et al., 2001, Zhuang et al., 2001). Due to the extreme fecundity of most penaeid species and the increased risks associated with inbreeding the ability to assign progeny to their family of origin is

Table 4. Summary of observed and expected Hemozygosity and heterozygosity, Polymorphism information content (PIC) and average heterozygosity per each microsatellite in this study.

Locus	Sampl e size	Obs_Hom	Obs_Het	Exp_Hom	Exp_Het	PIC	Ave_Het
CSCUPmo1	Wild	0.6667	0.3333	0.2138	0.7862	0.7600	0.6633
	Culture	0.8000	0.2000	0.4138	0.5862	0.5667	0.6633
CSCUPmo2	Wild	0.5333	0.4667	0.3678	0.6322	0.6111	0.5356
	Culture	0.6667	0.3333	0.5241	0.4759	0.4600	0.5356
CSCUPmo3	Wild	0.6000	0.4000	0.2230	0.7770	0.7511	0.7067
	Culture	0.6000	0.4000	0.3149	0.6851	0.6622	0.7067
CSCUPmo4	Wild	0.6667	0.3333	0.5057	0.4943	0.4778	0.4311
	Culture	0.6667	0.3333	0.6023	0.3977	0.3844	0.4311
CSCUPmo6	Wild	0.5333	0.4667	0.3609	0.6391	0.6178	0.5389
	Culture	0.5333	0.4667	0.5241	0.4759	0.4600	0.5389
CSCUPmo7	Wild	0.6000	0.4000	0.3816	0.6184	0.5978	0.4144
	Culture	0.8667	0.1333	0.7609	0.2391	0.2311	0.4144
Mean± St.Dve	Wild	0.6000±0.0596	0.4000±0.0596	0.3421±0.1096	0.6579±0.1096	0.6359±0.1059	0.5483±0.1185
Mean± St.Dve	Culture	0.6889±0.1241	0.3111±0.1241	0.5234±0.1537	0.4766±0.1537	0.4607±0.1486	0.5483±0.1185

Obs, observed; Hom, homozygosity; Het, hetrozygosity; Ave, average; PIC, polymorphism information content.

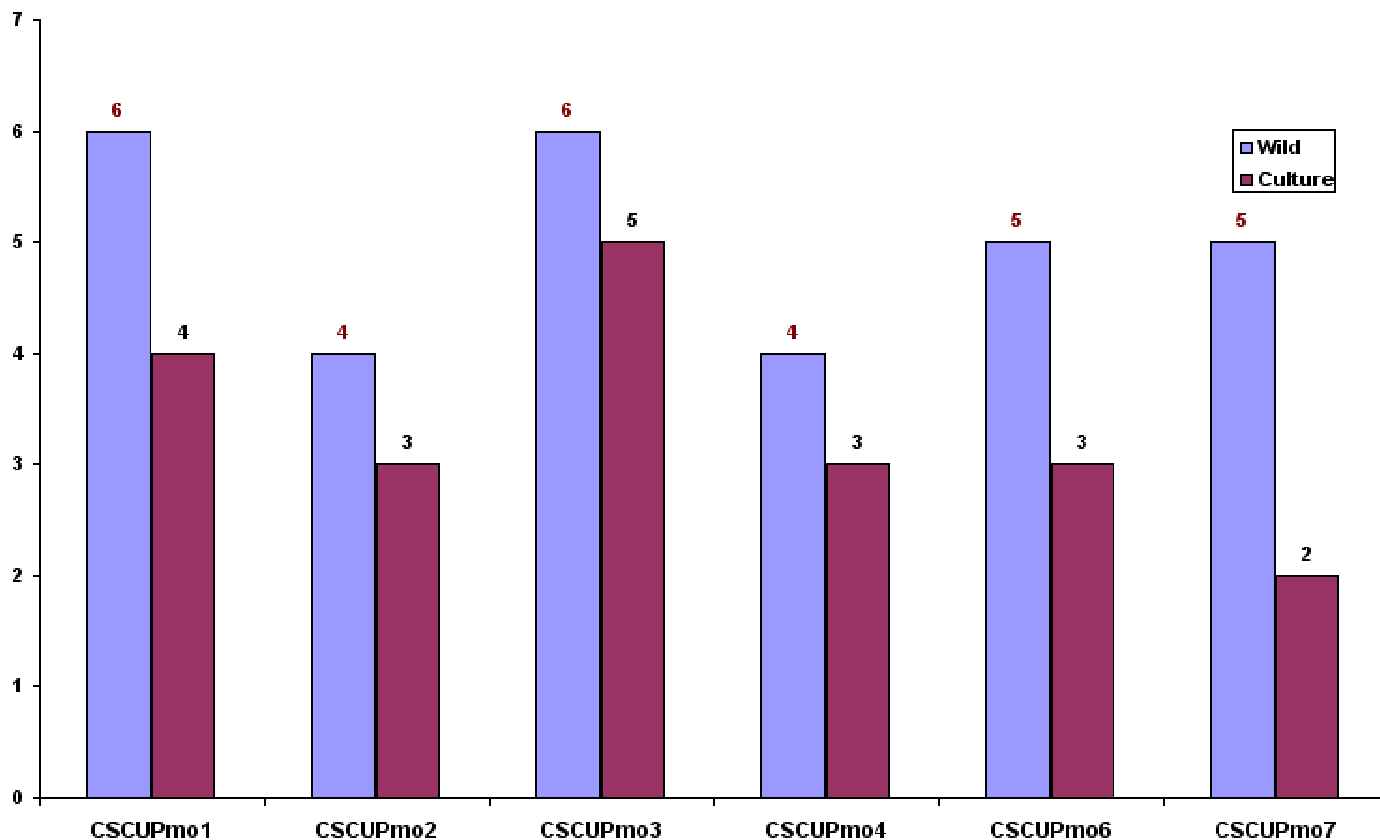


Figure 3. Observed number of alleles per each microsatellite in this study.

imperative to the long-term sustainability of selective breeding programs. The loss of genetic variability in shrimp brood stocks will happened through generations if breeders did not consider controlled mating and consequently inbreeding depression in small populations may contribute to the reduction of the survival, growth

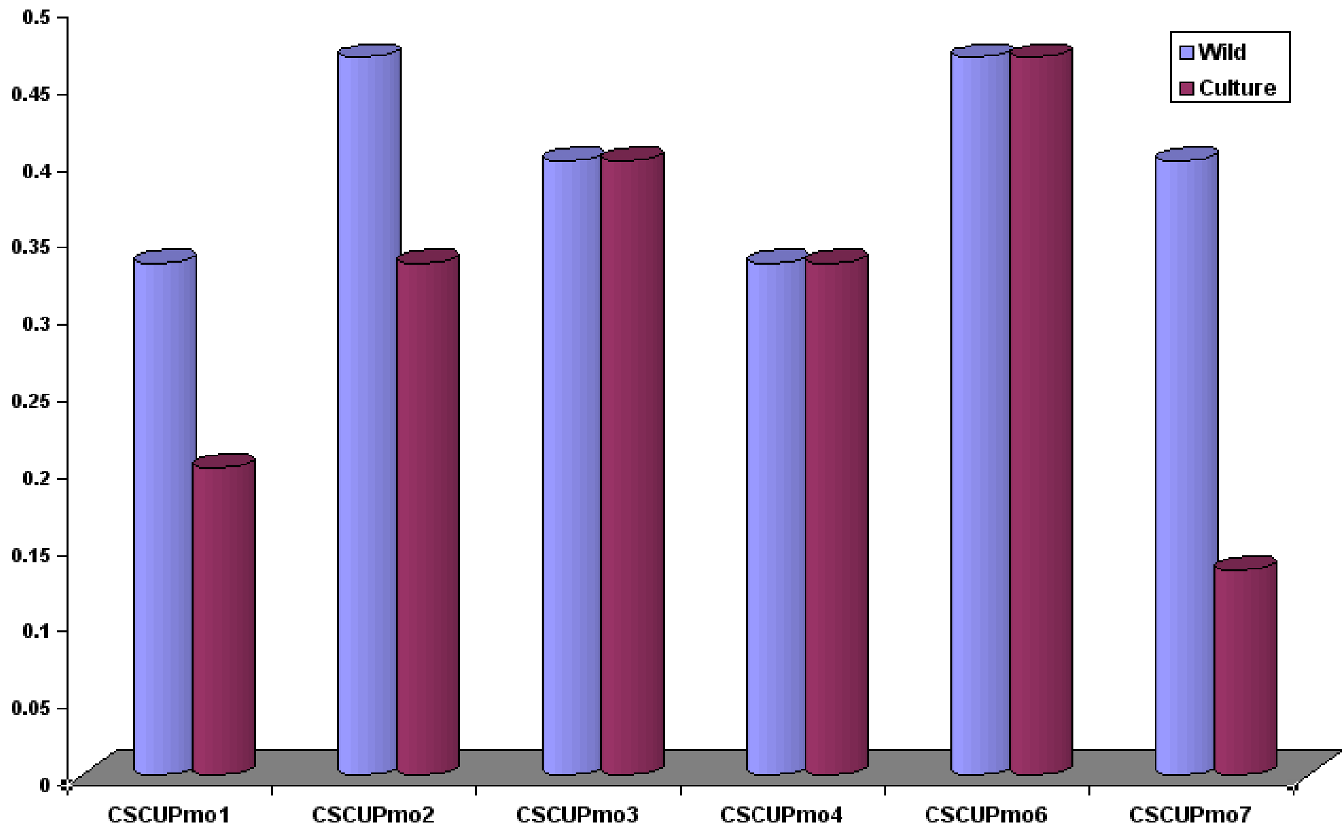


Figure 4. Summary of observed heterozygosity per each microsatellite in this study.

and reproduction rates (Gjedrem, 2005) and the capacity of the brood stocks to adapt to environmental changes (Sbordoni et al., 1986). The availability of brood stock of *P. monodon* mainly depends on the capture of the wild gravid females from the Malaysian water source. Unfortunately, the number of gravid females decreased year by year but on the other hand the price of wild gravid females increased every year. Therefore, to ensure a stable supply of seed of *P. monodon*, induce maturation and spawning in captivity are very essential. In this study, the number of alleles yielded by all microsatellites ranged from two to six alleles.

The highest and the lowest effective number of alleles were found in CSCUPmo1 (4.16) and CSCUPmo7 (1.3), respectively. The result of this study is comparable with other literatures. In this study, the MFA for CSCUPmo1, CSCUPmo2, CSCUPmo3, CSCUPmo4, CSCUPmo6 and CSCUPmo7 bp were 259, 165, 181, 242, 206 and 218 bp, respectively. Supungul et al. (2000) examined genetic diversity of *P. monodon* in Thailand by microsatellites (CUPmo1, CUPmo18, Di25, CSCUPmo1 and CSCUPmo2) using the same sample set as in this study. The average observed heterozygosity was relatively high in each geographic sample (0.71 to 0.82). Significant deviation from the Hardy–Weinberg expectation was observed in 19 of 25 possible tests owing to homozygote excess, even after the sequential Bonferroni procedure (Rice, 1989) was applied for multiple tests *($P< 0.001$).* Brood stock in this study showed lower genetic diversity value than wild population. However, with an observed heterozygosity (H_{obs}) below expectations, it would be necessary to introduce cross breeding among hatcheries to reduce the risk of inbreeding depression. Inbreeding increases homozygosity, which in some species can lead to reduced growth, viability and reproductive performance, and the benefits of heterosis have been demonstrated in shrimp (Goyard et al., 2008). Inbreeding has also been linked to biochemical disorders and deformities from lethal and sub-lethal recessive alleles (Dunham, 2004). Inbreeding can be avoided provided that a wide genetic variation is secured in the base or founder population, and further avoided if parentage of animals is known, and related-animal mating is avoided.

In conclusion, microsatellite markers analysis was able to characterize the genetic divergence between the brood stocks and wild population and could be helpful tools for defining better management strategies of these *P. monodon* (Fabricius) in Malaysia. The result of test and evaluating genetic diversity and inbreeding could enhance brood stock management in assisting shrimp breeders to minimize or avoid inbreeding, especially within genetic improvement programs in which inbreeding

can accumulate in few generations.

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