Characterization of Tri- and Tetranucleotide Microsatellites in the Black Tiger Prawn, *Penaeus monodon*

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ABSTRACT In order to characterize tri- and tetranucleotide microsatellites in the black tiger prawn, *Penaeus monodon*, a genomic library of *P. monodon* was screened with the repeats $(GAA)_n$, $(GATA)_n$, $(GGAT)_n$, $(GGAA)_n$ and $(CACC)_n$. In the 79 positive clones isolated, the repeats $(GATA)_n$ was found at the highest frequency, followed by the $(GAA)_n$ while the other sequence types were rare or not found. Sequencing of the microsatellite positive clones revealed that the predominant category of $(GAA)_n$ and $(GATA)_n$ microsatellites were perfect repeats, with the number of repeats ranging from 5 to 45 for $(GAA)_n$ and 4 to 16 for $(GATA)_n$. Primers were designed for seven microsatellite loci and 6 pairs of primers produce scorable PCR products. Analysis of polymorphism in wild *P. monodon* caught off the Gulf of Thailand revealed that all six microsatellite loci were highly polymorphic with 21 to 29 alleles and heterozygosities of 16 to 89%. Cross-amplification using these microsatellite markers on related penaeid shrimps suggested low level of sequence similarity in microsatellite regions among penaeids.

KEYWORDS: microsatellite, *Penaeus monodon*, black tiger prawn.

INTRODUCTION

Microsatellites are tandemly repetitive DNA sequences with very short nucleotide motifs (1-6 bp) and their repeat arrays are generally no longer than 300 bp.[1] They are abundant and distributed throughout the eukaryotic genome. Variation in the numbers of repeating units was demonstrated[2] and microsatellite polymorphisms can be assayed by the polymerase chain reaction (PCR).[3] Allelic variants of a microsatellite locus are codominant and show Mendelian inheritance. Microsatellites are being employed as genetic markers in a variety of organisms.[2,4] Their applications include genome characterization and mapping,[5,6] parentage and identity testing,[7,8] genetic differentiation and population studies.[9,10]

Microsatellite markers are being increasingly applied in fisheries and aquaculture.[11] The development of microsatellite markers have been reported in fish and marine invertebrate species including Atlantic salmon,[12] Atlantic cod,[13] seabass,[14] oyster[15] and lobster.[16]

In the commercially important marine shrimps, microsatellites have been characterized in *Penaeus vannamei*,[17,18] *P. japonicus*[19] and *P. monodon*.[20,21] Shrimp microsatellites, particularly the dinucleotide repeats, have been found to consist of very long repeat arrays which has resulted in difficulties in developing useful microsatellite markers. Tri- and tetranucleotide repeats, have become useful genetic markers in penaeids. These markers are easier to analyse as fewer stutter bands, characteristic of dinucleotide repeats, have been observed.

In this study, we further investigated sequence types and characteristics of tri- and tetranucleotide microsatellites in the black tiger prawn, *P. monodon* genome. We also report allelic variations and heterozygosities of 6 useful markers in wild population of *P. monodon* and examine whether these microsatellite loci are conserved in other penaeid species.

MATERIALS AND METHODS

Samples and DNA extraction

Wild *P. monodon* used to survey for polymorphism were taken from the east coast of Thailand (The Gulf of Thailand). Other penaeid species, *P. vannamei, P. japonicus, P. semisulcatus*, *P. mergiensis* and *P. indicus* which were tested for cross-amplification were also wild-caught animals. For cloning purposes, genomic DNA was extracted from the pleopod of a shrimp using the phenol/chloroform

extraction described in Sambrook *et al.*[22] DNAs used in genotyping were extracted using simple chelex method described by Walsh *et al.*[23]

Cloning and screening of tri- and tetranucleotide microsatellites

High molecular weight DNA was sonicated to yield approximately 500 bp fragments. The DNA fragments were treated with T4 polynucleotide kinase (Promega) and Klenow Polymerase (Promega) to repair ends. Approximately 150 ng of this DNA fragments was ligated into 50 ng of pUC18/*Sma*I (Pharmacia) at 16°C overnight. The ligation mixture was transformed into Epicurian Coli XL-10-Gold™ Ultracompetent cells (Stratagene). Transformed cells were grown up overnight on LB agar plates containing 50 µg/ml ampicillin with X-gal and IPTG.

Recombinant colonies were transferred onto Hybond-N$^+$ nylon membranes (Amersham) and hybridized with microsatellite probes as described by Moore *et al.*[19] The synthetic oligonucleotide probes, $(GATA)_6$, $(GGAT)_6$, $(GGAA)_5$, $(CACC)_5$ and a concatemer probe $(GAA)_n$ were used in library screening. The $(GAA)_n$ probe was labelled with [α-^{32}P]-dCTP by random priming method[22] while the oligoprobes were 5'-end labelling with [γ-^{32}P]-ATP and T4 polynucleotide kinase (Promega). The radiolabelled oligoprobes were hybridized to the colony-lifted membranes in a buffer containing 1M NaCl, 42 mM Tris-HCl pH 7.4, 1.2 mM EDTA, 0.2% SDS, 1% BSA and 0.1% sodium pyrophosphate, at Tm-5°C overnight but the $(GAA)_n$ concatemer were hybridized at 65°C overnight.

The filters probed with the end labelled oligonucleotides were washed in 2 x SSC, 0.1% SDS for 10 min at the hybridization temperature and twice for 10 min at room temperature in 0.2 x SSC, 0.1% SDS. Filters probed with $(GAA)_n$ were treated in the same way except all washes were performed at 65°C.

Sequencing and primer synthesis

Positive colonies were picked from the plates and grown in terrific broth.[22] Plasmid DNA was extracted by modified alkaline lysis/PEP precipitation,[22] sequenced using the ABI Prism™ Ready Reaction Dye Deoxy Cycle Sequencing Kit and analysed on a ABI 373 Automated Sequencer. Primers were designed from the unique sequences flanking microsatellites loci using the computer program MACVECTOR version 4.5.1 primer design software (International Biotechnologies Inc Kodak). The primers were synthesized by Geneworks, Adelaide, Australia.

PCR amplification

PCR conditions for each primer pair were optimized by testing at three different annealing temperatures: 50; 56; and 60°C, using the Robo Cycler Gradient Temperature Thermocycler (Stratagene). Other PCR conditions were fixed at 1 cycle of 95°C for 3 min, 30 cycles of denaturation at 95°C for 1 min, annealing for 2 min and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. One of each pair of primers was end-labelled with [γ-^{32}P]-ATP (Amersham) using T4 polynucleotide kinase. PCR was performed in a 20 µl reaction volume containing 5-50 ng of genomic DNA, 1X PCR buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 250 nM of each primer and 0.44 U of *Taq* polymerase. PCR products were separated on 6% denaturing acrylamide gels. Gels were dried and exposed to x-ray film overnight.

Data analysis

The number of alleles at each microsatellite locus, the proportion of individual samples that are heterozygous (direct count heterozygosity, H_{obs}) and the unbiased estimate of heterozygosity (H_{exp}), were assessed for each locus using a modified version of BIOSYS-1 program.[24] Hardy-Weinberg expectation test for each locus was a Markov chain "approximation to exact test" following Guo and Thompson[25] and carried out using the GENEPOP program.[26]

Results

Isolation of tri- and tetranucleotide repeats in *P. monodon*

The results of genomic library screening for microsatellite repeats $(GAA)_n$, $(GATA)_n$, $(GGAT)_n$, $(GGAA)_n$ and $(CACC)_n$ are summarized in Table 1. A total of 79 positive clones were found in the screening. The percentages of positive clones were 0.24% for $(GAA)_n$, 1.21% for $(GATA)_n$, 0.04% for $(GGAT)_n$, 0.05% for $(GGAA)_n$ and 0% for $(CACC)_n$. The results suggested that the trinucleotide $(GAA)_n$ and the tetranucleotide repeats $(GATA)_n$ were present in significant numbers in the *P. monodon* genome but the repeats $(GGAT)_n$, $(GGAA)_n$ and $(CACC)_n$ appeared to be rare or did not exist. Sequencing of those positive clones revealed 71 microsatellite containing clones and most of these clones contained more than one microsatellite array. According to Stalling *et al*,[5] a sequence is considered to be a microsatellite when

Table 1. Screening of the partial genomic library of *P. monodon* with various tri- and tetranucleotide probes.

Probe	Colonies screened	No of positive clones	Frequency (%)
$(GAA)_n$	9,900	24	0.24
$(GATA)_6$	4,200	51	1.21
$(GGAT)_6$	7,560	3	0.04
$(GGAA)_5$	1,900	1	0.05
$(CACC)_5$	1,900	0	0

the number of repeats is greater than 10 for mononucleotide, 6 for dinucleotide, 4 for trinucleotide, and 3 for tetranucleotide, pentanucleotide and hexanucleotide. A total of 163 microsatellite arrays were identified from 71 clones and among these microsatellites, 32 were $(GAA)_n$ and 36 were $(GATA)_n$ repeats. Seventy-seven dinucleotide microsatellites, including $(CT)_n$, (AT)n and $(GT)_n$, were found within those positive clones adjacent to the repeat arrays of either $(GAA)_n$ or $(GATA)_n$.

Microsatellite characteristics

The $(GAA)_n$ and $(GATA)_n$ microsatellites were classified into 3 categories, perfect, imperfect, and compound repeats as defined by Weber (1990).[27] Perfect repeats are uninterrupted stretches of repeat units, while imperfect repeats have one to three intervening bases with repeat sequence on either side. Compound repeats consist of several different repeat types and are separated by less than three bases. Compound repeats were further subdivided into perfect and imperfect sequences (Table 2). Perfect repeats were found at the highest percentages for both $(GAA)_n$ and $(GATA)_n$. For $(GAA)_n$, 66% of the repeats were perfect microsatellite, while 53% of those $(GATA)_n$ isolated were perfect microsatellites. Compound repeats were also found at high percentage (42%) for (GATA)n while only 9% of $(GAA)_n$ were compound repeats. A range of repeat numbers were 5-45 for $(GAA)_n$ and 4-16 for $(GATA)_n$. The maximum repeat number may be underestimated because there were 4 clones which had the cloning sites occurred within the repeats.

Table 2. Repeat range and percentage of perfect, imperfect and compound repeats in *P. monodon* microsatellites.

Repeat	Total no	Range of repeat no	Category of Repeat						
			Perfect		Imperfect		Compound		
			No	%	No.	%	No of perfect	No of imperfect	%
$(GAA)_n$	32	5-45	21	66	8	25	1	2	9
$(GATA)_n$	36	4-16	19	53	2	5	7	8	42

Table 3. Repeat sequences, PCR primers, and optimized annealing temperature for 6 microsatellite loci in *P. monodon*.

Locus	Repeat sequences	Primer sequences*	Annealing Temp (°C)
CSCUPmo1	$(GAA)_{43}$	F = ATGATGGCTTTGGTAAATGC	
		R = CGTACTTCCTCTTCATAGGTATC	56
CSCUPmo2	$(ATCT)_{12}(TA)_{10}(TAGA)_3$	F = CCAAGATGTCCCCAAGGC	
		R = CTGCAATAGGAAAGATCAGAC	56
CSCUPmo3	$(ATCT)_{12}(AT)_9T(GT)_9$	F = TGCGTGATTCCGTGCATG	
		R = AGACCTCCGCATACATAC	56
CSCUPmo4	$(CT)_{10}TG(CT)_{17}(ATCT)_{10}$	F = TTTCTTTCTTCTCGTGATCCC	
		R = GACGGCATGAGGAATAGAGG	52
CSCUPmo6	$(GATA)_6(GA)_{16}$	F = TAGTGTTACTCAGGTGCAGC	
		R = GCGTGTATTTGTGATTTCAC	56
CSCUPmo7	$(CT)_{15}(ATCT)_9$	F = ACGAATGAATGCGGTGGTGC	
		R = TCGGTGCCCAGTTGTATGAGAG	56

* F indicates forward; R, reverse.

However, most of the repeat numbers for the two microsatellite types were less than 15 repeats.

Many compound repeats contain more than 2 different repeat types separated by 1 to 3 bases indicated the complexity of *P. monodon* microsatellites. $(GATA)_n$ microsatellites are frequently associated with dinucleotides $(AT)_n$ and $(CT)_n$ and tetranucleotide $(CATA)_n$ repeats.

Primer design and microsatellite polymorphism

Although most tri- and tetranucleotide microsatellites exhibited less than 15 repeats, they were often associated with other dinucleotide microsatellites which contained very long repeat arrays. Moreover, many compound microsatellites consisted of several different repeat types and some microsatellites were adjacent to the cloning sites so unique sequences flanking the repeats for primer design were not available. Only 7 pairs of primers were designed, 1 from $(GAA)_n$ and 6 from $(GATA)_n$ repeats. Six primers produced scorable PCR products with size in the expected range. The repeat type, primer sequences and annealing temperature for each microsatellite locus are shown in Table 3. Examples of amplified alleles of unrelated individuals using CSCUPmo1 (alleles 224-326 bp) and CSCUPmo2 (alleles 137-217 bp) were shown in Figs 1 and 2.

DNA samples from wild *P. monodon* caught off the Gulf of Thailand were analysed at the six microsatellite loci. All six loci were polymorphic with 21 to 29 alleles and heterozygosities of 16 to 89% (Table 4). A comparison of the observed heterozygosities with expected values suggest that there was a deficiency of heterozygotes and consequently all loci except the locus CSCUPmo4, were not in Hardy-Weinberg equilibrium (p<0.05).

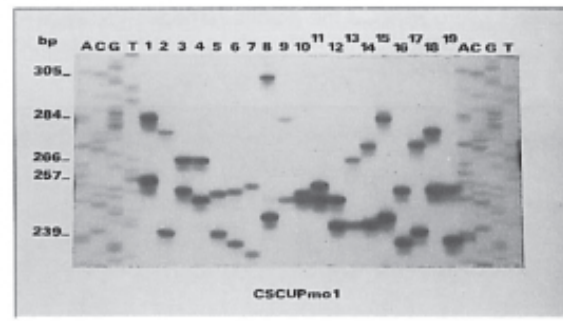

Fig 1. PCR amplified alleles for CSCUPmo1 locus from unrelated *P monodon*.
Lanes 1-19: 19 unrelated animals
The size of PCR products were estimated using M13 sequencing ladder (lanes A, C, G, and T).

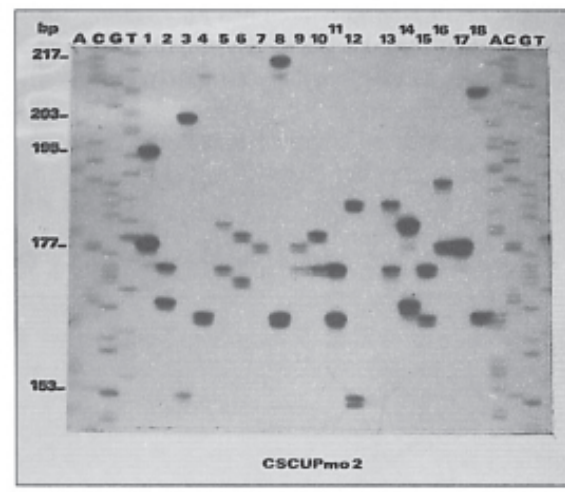

Fig 2. PCR amplified alleles for CSCUPmo2 locus from unrelated *P monodon*.
Lanes 1-18: 18 unrelated animals
The size of PCR products were estimated using M13 sequencing ladder (lanes A, C, G, and T).

Table 4. Allele size and heterozygosity at 6 microsatellite loci detected by PCR amplification.

Locus	Sample size	Observed alleles	Size range (bp)	H_{obs}*	H_{exp}#	H-W † (p value)
CSCUPmo1	51	29	224-326	0.73	0.96	<0.0001
CSCUPmo2	51	28	137-217	0.78	0.94	0.0024
CSCUPmo3	49	27	135-223	0.63	0.93	<0.0001
CSCUPmo4	45	21	206-256	0.89	0.89	0.2138
CSCUPmo6	49	27	166-242	0.69	0.96	<0.0001
CSCUPmo7	43	21	172-234	0.16	0.94	<0.0001

*Observed heterozygosity; #Expected heterozygosity,
†Probability of agreement to Hardy-Weinberg equilibrium.

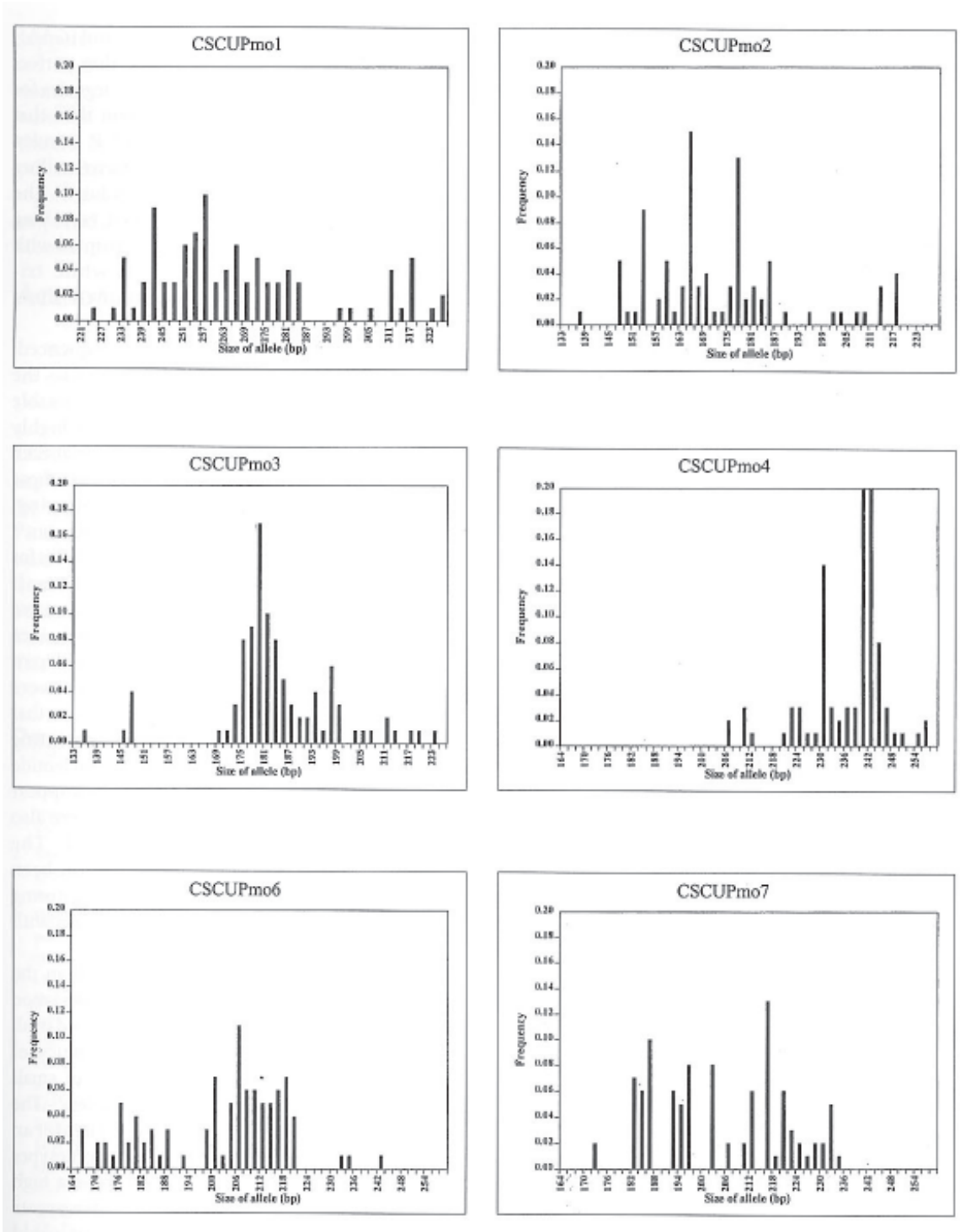

Fig 3. Distribution of allele frequencies in wild *P. monodon* from The Gulf of Thailand at six microsatellite loci: CSCUPmo1; CSCUPmo2; CSCUPmo3; CSCUPmo4; CSCUPmo6; and CSCUPmo7.

The six microsatellite loci varied in allele size range from 135-326 bp. The allele frequency distributions for the six microsatellite loci in wild *P. monodon* are shown in Fig 3. All loci showed wide variation in allele size range. Allele size differences of 3 bp were found in CSCUPmo1 and 2 bp differences were found in the other 5 loci. There were gaps in the range of allele sizes, particularly at CSCUPmo3, there was a jump from 147 to 169 bp. The frequencies of the common alleles did not exceed 20% in any one of the loci.

Conservation of microsatellite markers

Conservation of microsatellites was examined by testing for amplification of the six microsatellite loci in five penaeid species: *P. vannamei, P. japonicus, P. semisulcatus*, *P. merguiensis* and *P. indicus*. The PCR conditions used were those optimal for amplification of microsatellite loci from *P. monodon* and thus may not be optimal in the other species. Primers from *P. monodon* microsatellite loci CSCUPmo1, CSCUPmo2 and CSCUPmo6 weakly amplified five other penaeid species (five individuals for each species) yielding faint bands of amplified alleles suggesting at least partial conservation of the priming sites (data not shown). The CSCUPmo3 and CSCUPmo7 primers amplified non-specifically in other penaeids yielding unscorable DNA patterns while no product was detected after PCR with the CSCUPmo4 primers.

Discussion

Tri- and tetranucleotide microsatellites have been demonstrated to be highly polymorphic and are stably inherited in the human genome.[28] They are becoming increasingly popular markers because the allele differences are easier to distinguish than those of dinucleotide repeats and less stutter bands have been observed in the amplification products. In this study, the abundance of different tri- and tetranucleotide repeats in the *P. monodon* genome were examined. By screening the genomic library with 5 repeat types and sequencing of positive clones, only the repeats $(GATA)_n$ and $(GAA)_n$ were found at significant amounts. Xu *et al*[21] have found various sequence types of tri- and tetranucleotide microsatellites in *P. monodon* by direct sequencing of recombinant clones without probe screening. The simple repeat$(GATA)_n$ was first identified and isolated from snake satellite DNA[29] and subsequently it has been found throughout the eukaryotic genomes. The low frequency of positive clones found in the screening suggests that enrichment of microsatellite sequences prior to screening would be useful.[30]

Classification of the repeats $(GATA)_n$ and $(GAA)_n$ based on Weber's criteria,[27] revealed that perfect microsatellites were the predominant categories for both types of repeats. This was different than that found in the dinucleotide repeats of *P. mondon* reported previously[20] where imperfect microsatellites were found to be the most abundant. The dinucleotide microsatellites contained very long repeat arrays and most often were interrupted with one to three bases on either side while tri- and tetranucleotide repeats contained short uninterrupted repeats.

From the 71 microsatellite clones sequenced, only 7 primer pairs were designed due to the complexity of microsatellite arrays. Six usable markers were obtained. All six loci were highly polymorphic exhibiting more than 20 alleles at each locus. The high level of polymorphism makes these microsatellite loci ideal for genome mapping, determining of parentage and pedigree analysis.[11] The allele differences between consecutive alleles for all loci depended on the repeat type. For CSCUPmo1, which was a perfect $(GAA)_n$ repeat, consecutive alleles differ by 3 bp. For the other loci, which were compound repeats of $(GATA)_n$ linked to different types of dinucleotide repeats, the alleles were different by multiples of 2 bases. This suggests that the polymorphism detected at these loci is occurring both within the dinucleotide and the tetranucleotide repeat arrays. The stutter bands which often appear on the dinucleotide microsatellite alleles were also observed in all loci except CSCUPmo1. The appearance of shadow or stutter bands has been shown to be due to slipped-strand mispairing during PCR[31] and the stutter bands tend to decrease with increasing unit length.[28]

Observed heterozygosities were lower than the expected values in all six loci and significant deviation from Hardy-Weinberg proportion was observed in all except the CSCUPmo4 locus. The deficiency of heterozygotes observed could be a result of small sample size[32] or the presence of null alleles.[33] The sample size of 50, which is normally adequate for an allozyme study, may not cover all possible genotypes generated by microsatellite loci which have a high number of alleles per locus. The extreme heterozygote deficiency found at the CSCUPmo7 locus could possibly be caused by mis-scoring of the stutter alleles or the presence of null alleles.

The weak amplification obtained with *P. monodon* primers when used with other penaeid species suggests a low level of sequence similarity in

microsatellite regions among penaeid shrimps. This supports the finding of Moore *et al* [19] that primers are likely to be largely species specific among a number of penaeids. However, the usefulness of *P. monodon* microsatellites in closely related species should be further investigated by changing the primer binding sites in each locus. Xu *et al*[21] have reported that 3 out of 10 *P monodon* microsatellites amplified alleles in *P.vanamei* indicating the potential of some microsatellites for cross-species amplification.

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