African Journal of Biotechnology Vol. 10(74), pp. 16715-16719, 23 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2216
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Population of bottleneck and microsatellite: An Analysis Based on genetic diversity of Wild Tiger Shrimp *Penaeus monodon* (Fabricius) in Malaysia

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Accepted 21 October, 2011

In the present study, six microsatellite markers, namely, CSCUPmo1, CSCUPmo2, CSCUPmo3, CSCUPmo4, CSCUPmo6 and CSCUPmo7 were used to monitor genetic variation in the wild tiger shrimp, *Penaeus monodon* (Fabricius). The number of alleles examined by all the microsatellites yielded the monitoring ranges of 2 to 6 alleles. Moreover, the highest and lowest heterozygosity were found to be at a significant level of 0.33 in CSCUPmo1 and at a significant level of 0.47 in CSCUPmo2, respectively. These observations indicated the existence of substantial genetic diversity in *P. monodon* in Malaysia. None normal 'L' shaped distribution of mode-shift test, significant heterozygote excess on the basis of different models, as revealed from Sign, standardized differences and Wilcoxon sign rank tests as well as significant M ratio value suggested that there was recent bottleneck in the existing population, providing important information for shrimp breeders.

Key words: Genetic bottleneck, *Penaeus monodon*, microsatellite, heterozygote.

INTRODUCTION

Monitoring of genetic variation with molecular markers across generations is recommended to reveal changes in variability that could be caused by events such as genetic drift, bottleneck, inbreeding or selection (Nahavandi et al., 2011). A genetic bottleneck is an evolutionary event by which a significant percentage of a population or species could be prevented from reproduction. Populations of bottlenecks occur when a population undergoes a temporary, high reduction in number (Keller et al., 2001). This can cause a reduction in genetic diversity, both in terms of heterozygosity and allelic diversity. In addition to bottleneck effects, the resulting small populations are often prone to high levels of inbreeding, since mate choice is reduced and individuals are more likely to mate with kin (Hedrick and Kalinowski, 2000). The shrimp industry in Malaysia, have shown a rapid development since the early 1980s which is said to have been motivated by the so-called successes that had previously been experienced in neighboring countries, such as Thailand, Indonesia and the Philippines during those years. However, Malaysia was not yet one of the major producers of cultured marine prawn in the world.

Microsatellites, or simple sequence repeats (SSRs) have been discovered in 1981, and are tandem repeated motifs of 1-6 nucleotides found in all prokaryotic and eukaryotic genomes (Goldstein et al., 1999). Nevertheless, dinucleotide repeats have dominated other

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types of microsatellites or SSRs, of which mono- and tetranucleotide repeats as well as trinucleotide repeats are the least dominants. Repeats of five (penta-) or six (hexa-) nucleotides are usually found amongst microsatellites or SSRs. 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).

The objective of this study was to analyze population of bottleneck and microsatellite based on the genetic diversity of wild tiger shrimp, *Penaeus monodon* (Fabricius) in Malaysia. In this study, two-phased model (TPM) together with Bayesian approach and Wilcoxon test (as recommended by the authors for microsatellite data) was employed to test the significance of the results after permuting the data. Moreover, our methodological application was based on sample and DNA extraction approaches.

MATERIALS AND METHODS

Samples and DNA extraction

A total of fifty individuals of wild *Penaeus monodon* from the both sexes were randomly selected to be investigated for polymorphism (Phenotype of wild *Penaeus monodon* (A) and collection sites in Sabah state, Malaysia (B) are given in figure 1). All the samples were immediately labeled; packaged using aluminum foil and kept inside ice flask till they reached to the laboratory. Genomic DNA was extracted from the pleopod of the mature shrimps using a commercial Kit (Qiagen, USA) according to the instruction of the manufacturer. The Purity of the extracted DNA was assessed by calculating the OD_{260}/OD_{280}nm ratio using a spectrophotometer (Genomic DNA extracted from shrimp tissue are given in Figure 2).

PCR amplification of the genes

PCR amplification was carried out in 25 µl volumes comprising of 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.01 mM of each primer, 50 ng of genomic DNA and 0.2 u Taq DNA polymerase. The sequences of the forward and reverse primers for the amplification of the microsatellite are given in Table 1. Amplification was carried out using a Termocycler (Ependorff, Germany). After an initial denaturation for 3 min at 94°C, the samples were subjected to 34 cycles of denaturation for 45 s at 94°C, annealing for 1 min at temperatures specific for the primers and extension at 72°C for 45 min and a final extension at 72°C for 10 min. The products were visualized on metaphor gel (4%) stained with 0.5 mg/l ethidium bromide for 20 min.

Statistical analysis

After PCR, result of reading gel was put in excel sheet; allele binning was used for minimums genotyping error originated from the 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 following the work of Yeh et al. (1999).

Furthermore, for the analysis of the probability of a recent reduction in the sample size, data file format was used in further analyses for which it had been performed by using bottleneck following Cornuet and Luikart (1996). This type of analysis is based on the hypothesis test that the allelic diversity hypothesis (H_E) is reduced faster than the heterozygosity given the hypothesis (H_O) samples experience of recent reduction of their effective sample size (Keller et al., 2001).

In order to detect the recent effective sample size reductions, the program bottleneck uses the allelic frequencies and it computes for each sample of size and therefore, for each locus, the distribution of the heterozygosity from the observed number of alleles (k), given the sample size (n) under the assumption of mutation-drift equilibrium is expected to occur. This distribution is obtained through simulating the coalescent process of n genes under three possible mutation models, the IAM (Infinite allele model), TPM (two-phased model, both IAM and SMM in different percentages used in simulation) and the SMM (stepwise mutation model) using either genetic distance or Bayesian approaches. This in turn enables the computation of the average (Hexp), which is compared with the observed heterozygosity to establish whether or not there is a heterozygosity excess or deficit at this locus.

In addition, the standard deviation (SD) of the mutation-drift equilibrium distribution of the heterozygosity is used to compute the standardized differences for each locus ((HO-HE)/SD). The distribution obtained through simulation enables also, the computation of a P-value for the observed heterozygosity. Once all loci available in a sample of sample size were processed, the three statistical tests: Sign test, Wilcoxon test and standardized differences test, were performed for each mutation model and the allele frequency distribution was established in order to see whether it is approximately L-shaped (as expected under mutation-drift equilibrium) or not (recent bottlenecks provoke a mode shift).

RESULTS AND DISCUSSION

Microsatellite polymorphism was studied in the population of bottleneck using the extracted genomic DNA (Figure 2) and the six pairs primers are listed in Table 1. The results show that the number of alleles yielded by all micro satellites ranged from 2 to 6 alleles. The highest and lowest effective number of alleles were found in CSCUPmo1 (4.16) and CSCUPmo7 (1.3) with the most frequent alleles (MFA) of 259 and 218 bp, respectively. The bottleneck analysis revealed a statistically significant result, indicating a recent bottleneck for any of the wild shrimp analyzed. The null hypothesis "the population is at migration-drift equilibrium" could not be rejected as the probabilities that had been estimated were lower than significant values of 0.05. These results are comparable with that of other studies. For example, Benzie (2000) taking this example which occurred in *P. japonicus*, highlighted the potential damaging effects of uncontrolled inbreeding and the need to maintain reasonably high levels of effective population sizes. A strict correlation between loss in genetic variation and shrimp production performance that would thereby reduce their genetic diversity, that is, adaptive potential, relative to their wild counterparts is well documented (Dumas and Ramos, 1999; Ibarra, 1999; Sbordoni et al., 1986; Harris et al., 1990; Xu et al., 2001; Zhuang et al., 2001).

Supungul and colleagues (2000) examined genetic diversity of *P. monodon* in Thailand by using the microsatellites CUPmo1, CUPmo18, Di25, CSCUPmo1 and CSCUPmo2 based on the same sample set as in the present study. However, the average observed hetero-

Table 1. List of primers for polymorphism analysis of microsatellite.

Locus	Motif	Primer sequence	Reported allele size	Annealing
CSCUPmo1	$(GAA)_{43}$	F = ATGATGGCTTTGGTAAATGC R = CGTACTTCCTCTTCATAGGTATC	224-326	56
CSCUPmo2	$(ATCT)_{12}(TA)10(TAGA)_3$	F = CCAAGATGTCCCCAAGGC R = CTGCAATAGGAAAGATCAGAC	137-217	56
CSCUPmo3	$(ATCT)_{12}(AT)_9T(GT)^9$	F = TGCGTGATTCCGTGCATG R = AGACCTCCGCATACATAC	135-223	56
CSCUPmo4	$(CT)_{10}TG(CT)17(ATCT)_{10}$	F = TTTCTTTCTTCTCGTGATCCC R = GACGGCATGAGGAATAGAGG	206-256	52
CSCUPmo6	$(GATA)_6(GA)_{16}$	F = TAGTGTTACTCAGGTGCAGC R = GCGTGTATTTGTGATTTCAC	166-242	56
CSCUPmo7	$(CT)_{15}(ATCT)_9$	F = ACGAATGAATGCGGTGGTGC R = TCGGTGCCCAGTTGTATGAGAG	172-234	56

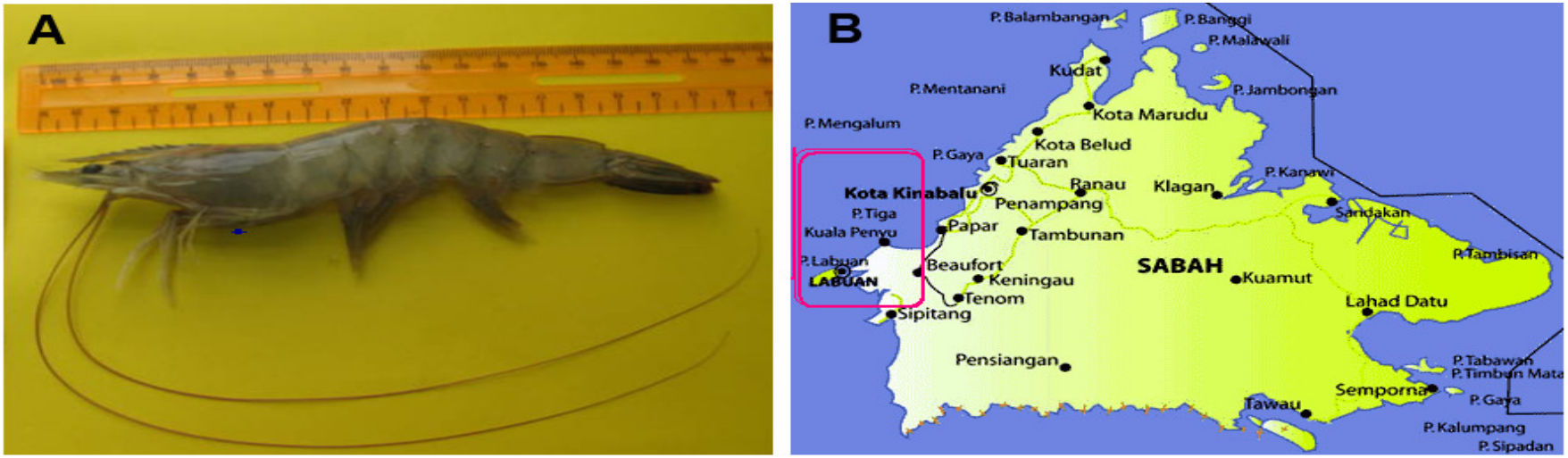

Figure 1. Phenotype of wild *P. monodon* (A) and collection sites in Sabah, Malaysia (B).

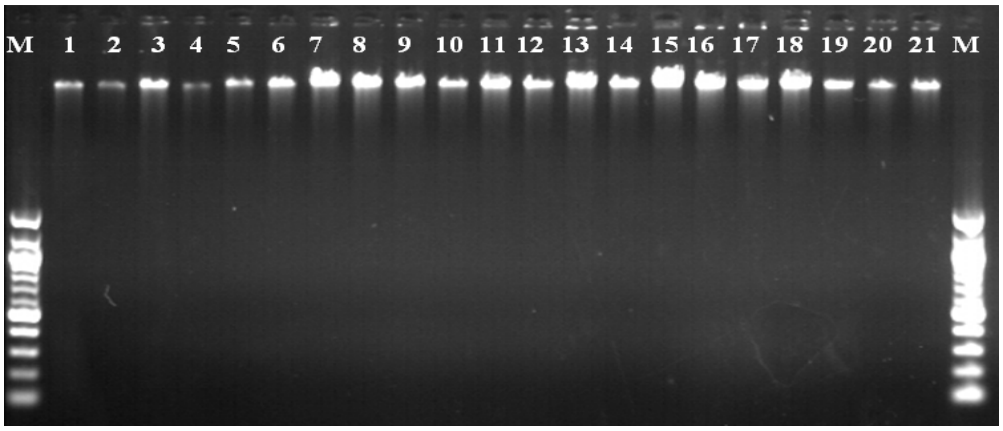

Figure 2. Genomic DNA extracted from shrimp tissue. Genomic DNA was extracted from the muscle tissue of the samples using a conventional kit. DNA was visualized on 4% metaphor gel. M: 100 bp DNA ladder (Fermentas); lanes 1 to 21: genomic DNA.

Table 2. Bottleneck analysis results.

Locus	Number	Observed		Under the IAM				Under the TPM				Under the SMM			
		ko	He	Heq	S.D.	DH/sd	Prob	Heq	S.D.	DH/sd	Prob	Heq	S.D.	DH/sd	Prob
Loc 1	30	6	0.33	0.679	0.107	-3.255	0.009	0.734	0.082	-4.914	0	0.769	0.058	-7.615	0
Loc 2	30	4	0.47	0.532	0.145	-0.431	0.284	0.59	0.115	-1.05	0.153	0.633	0.098	-1.656	0.072
Loc 3	30	5	0.333	0.621	0.117	-2.466	0.019	0.67	0.1	-3.361	0.011	0.715	0.072	-5.312	0.001
Loc 4	30	3	0.312	0.422	0.16	-0.686	0.314	0.457	0.145	-0.999	0.197	0.505	0.126	-1.525	0.103
Loc 5	30	3	0.432	0.418	0.16	0.089	0.487	0.466	0.144	-0.236	0.367	0.503	0.128	-0.554	0.26
Loc 6	30	2	0.408	0.243	0.163	1.012	0.222	0.265	0.163	0.878	0.259	0.287	0.165	0.737	0.309

Assumptions: All loci fit IAM, mutation-drift equilibrium; Expected number of loci with heterozygosity excess: 3.25; 4 loci with heterozygosity deficiency and 2 loci with heterozygosity excess; probability: 0.26804. Assumptions: All loci fit TPM, mutation-drift equilibrium; Expected number of loci with heterozygosity excess: 3.45; 5 loci with heterozygosity deficiency and 1 loci with heterozygosity excess; probability: 0.05283. Assumptions: All loci fit SMM, mutation-drift equilibrium; Expected number of loci with heterozygosity excess: 3.54; 5 loci with heterozygosity deficiency and 1 loci with heterozygosity excess; probability: 0.04559.

zygosity was relatively high in each geographic sample which was between 0.71 and 0.82. The result was of course a significant deviation from the Hardy-Weinberg expectation which had been 19 out of 25 possible tests owing to homozygote excess that had been observed, even after the sequential procedure of Bonferroni which was applied for multiple tests at a significant level of ($P<0.001$).

All the loci were subjected to statistical analysis using the three models of microsatellite evaluation, IAM, stepwise mutation model (SPM) and TPM to test whether the populations have undergone recent genetic bottleneck. Table 2 summarizes the results of the analysis. Four loci with heterozygosity deficiency (locus 1 to 4) and two with heterozygosity excess (loc 5 and 6) was detected using IAM model, while in TPM and SMM model, heterozygosity excess was only observed in loci 6. These observations showed the existence of bottleneck in the population.

When a population goes through a bottleneck process,

many low frequency alleles may be eliminated, or contribute little to heterozygosity. Thus, in a bottleneck of short duration, this process could severely reduce the number of alleles (that is, by eliminating rare alleles) without having much effect on heterozygosity (Norris et al., 1999). These types of implication were discussed by Goyard et al. (2003) who considered the number of spawners participating in the maintenance of the populations of *Litopenaeus stylirostris* to be very small and were likely to increase inbreeding. Wolfus et al. (1997) also considered that an inbred population could have resulted from the genetic contribution of only two alleles. Thus, inbreeding increases homozygosity, which in some species can lead to reduced growth, viability and reproductive performance, but 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). However, inbreeding can be avoided when a wide genetic variation is secured in the base or founder population, and could further be avoided if parentage of animals is known, and if related-animal mating is avoided.

The loss of genetic variability in shrimp brood stocks would have 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 and reproduction rates as well as the capacity of the brood stocks to adapt to environmental changes (Gjedrem, 2005; Sbordoni et al., 1986). Population bottleneck and founder effects may cause a reduction in the number of alleles, a distorted distribution in numbers of molecular differences among alleles, and an increased linkage disequilibrium, resulting in a serious loss of heterozygosity (Hart and Clark, 1997; Pirchner, 2000).

Overall, the present study showed heterozygosity deficiency and excess in the investigated loci, indicating the existence of bottleneck in the population.

ACKNOWLEDGMENTS

The authors would like to thank KPT for providing financial support to carry out this research work and University Putra Malaysia (UPM) (Institute of Bioscience) for the technical assistance and providing research facilities.

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