Genetic diversity among three Indian populations of black tiger shrimp (*Penaeus monodon* Fabricious, 1798) using microsatellite DNA markers

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ABSTRACT

Genetic variation is an essential factor while selecting superior stocks for breeding programs. Eight microsatellite loci (*TUZX pm2.27, TUZX pm2.28, TUZX pm2.36, TUZX pm2.69, TUZX pm4.84, TUZX pm4.87, TUZX pm6.1* and *TUZX pm4.9*) were used for molecular characterisation of *Penaeus monodon* collected from three different locations in India, *viz*., Kakinada and Chennai on the east coast and Mumbai on the west coast and all the loci were found to be highly polymorphic. The number of alleles and genotypes across the loci ranged from 5 to 23 and 42 to 98, respectively. The observed heterozygosity among the populations across the loci ranged from 0 to 0.1678. The overall F_{ST} value was found to be 0.021 (<0.05), which implies a low degree of population differentiation in the species. On the basis of pair-wise allelic differentiation and UPGMA dendrogram, the investigated samples from three locations were allocated in to two distinct populations composed of east coast and west coast populations.

Keywords: Breeding, Genetic variation, Microsatellite, *Penaeus monodon*

Introduction

Penaeid shrimps are an ecologically diverse group of species and important resources for fisheries and aquaculture worldwide (Chatti *et al*., 2008). Black tiger shrimp, *Penaeus monodon,* is one of the most economically important penaeid species, widely distributed in the Indian Ocean and Western Pacific Ocean (Holthius, 1980). In India, culture of this species has shown rapid development during the last two decades. India's vast coastal belt and favourable environment for culture led to the rapid expansion of shrimp farming. Consequently, in the recent years, shrimp hatcheries are on the rise to meet the ever increasing demand for seeds, which further necessitated the demand for female brooders. The lack of knowledge on the captive breeding of *P. monodon* has led to heavy exploitation of wild brooders. Therefore, basic knowledge about genetic diversity and population differentiation of *P. monodon* is vital to overcome the selection of an inbred broodstock from wild population for breeding programs (Jayabhand *et al*., 1998). Genetic variation is one of the essential components for selection programs that aim to enhance ecologically or economically important traits (Lundrigan *et al*., 2005). To estimate the genetic variation, various methods such as morphometry, truss network analysis, biochemical and molecular polymorphisms have been proposed, and these are helpful in stock identification of finfish and shellfishes (Shetty, 2004).

Microsatellites are widely used as genetic markers in shrimp species such as *P. monodon* (Xu *et al*., 1999; Mandal *et al*., 2012), *Litopenaeus vannamei* (Garcia *et al*., 1994) and other shrimp species in the world. In India, the application of microsatellites to analyse the genetic variation and population differentiation in several fishes has been reported. However, reports on such microsatellite based analysis in wild *P. monodon* are limited (Mandal *et al*., 2012). Therefore, in the present study, the *P. monodon* samples from different geographical locations were collected in order to genetically characterise the wild populations using microsatellite DNA markers.

Materials and methods

Collection of samples

Samples of wild *P. monodon* were collected from three locations in India: Chennai and Kakinada from east coast and Mumbai from west coast (Fig. 1).   A total of

164 individuals (55 each from Kakinada and Mumbai and 54 from Chennai) were randomly collected from fish landing centres during the same period. The pleopods and uropods of each specimen were collected and stored in absolute ethanol and transported individually to the laboratory and stored at -20 °C until DNA extraction.

DNA extraction and PCR amplification

The total genomic DNA from the pleopods and uropods of the samples was isolated by SDS-Phenol-Chloroform
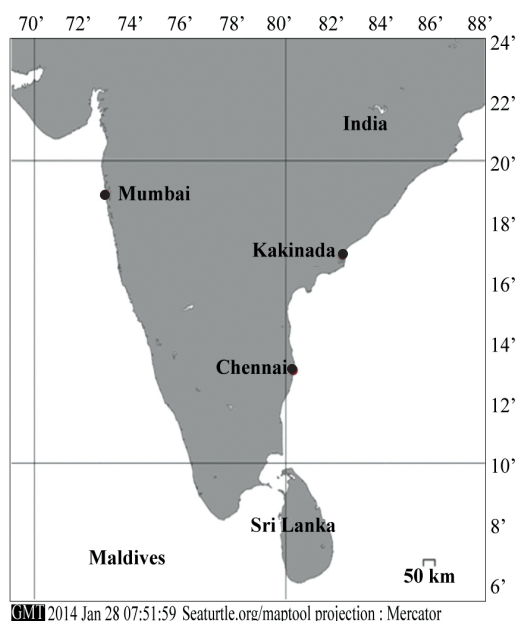

Fig. 1.   Collection sites of *P. monodon* samples in the east and west coasts of India

method described by Sambrook *et al*. (2001). The quality of DNA was checked by agarose gel electrophoresis and quantity was measured using a Biophotometer (Eppendorf, Germany). For molecular characterisation, eight species specific primer sets were used for amplification of microsatellite loci such as *TUZX Pm2.27*, *TUZX Pm2.28, TUZX Pm2.36, TUZX Pm2.69, TUZX Pm4.84, TUZX Pm4.87, TUZX Pm6.1* and *TUZX Pm4.9* (Xu *et al*., 2001). The details of the primers used in the study are given in Table 1. All the PCR reactions were performed using PCR Express Thermal Cycler (Thermo Hybrid, U. K). The thermal cycler was programmed for 30 cycles of denaturation at 94°C for 1 min, annealing for 30 sec with primer specific annealing temperature (Table 1), and extension at 72°C for 1 min. The initial denaturation was carried out at 94°C for 3 min and final extension at 72°C for 5 min.

Microsatellite band scoring

The PCR amplified products were separated on 8% non-denaturing polyacrylamide gels (PAGE) and stained with ethidium bromide in water. The stained gels were visualised with ultraviolet light and documented using a gel documentation system with Gene Snap software (Syngene, USA). The size of the separated alleles was determined by comparison with 50 bp DNA marker (Fermentas) using Gene Tool software (Syngene, USA). The primary alleles were selected based on its expected band size and intensity. The alleles, such as homozygous, heterozygous and null were scored based on number of bands of the expected size.

Table 1. Microsatellite primers and optimum annealing temperatures used in the study

Locus	Primers	Annealing temperature (°C)	Accession No.
TUZX pm2.27	F-5' CTCTCTGTCCGTGTGAGC 3' R-5' CATGTCAAAACGAGGAGGT 3'	50	AF077551
TUZX pm2.28	F-5' GTCCCTCTAATGATGTCT 3' R-5' CGCACTTCAGTGCAGGGC 3'	50	AF077552
TUZX pm2.36	F-5' AACGCGTGATGAAACACC 3' R-5' ATCGAGTTCGGTCGAAGA 3'	50	AF077554
TUZX pm2.69	F-5' CAAGTAAGAAGTGCCTCC 3' R-5' CAGTCTAAAAGCTTGCCT 3'	50	AF077557
TUZX pm4.84	F-5' GCTCAGCACATTTCCGGC 3' R-5' GGGATTTGAGGACGATGG 3'	50	AF077595
TUZX pm4.87	F-5' GGAAATCTGGCTAG 3' R-5' GGGATTTGAGGACGATGG 3'	50	AF077598
TUZX pm6.1	F-5' AAAACGGCGGTTGCTTCTC 3' R-5' CCTCGGTATGGTGATGACATG3'	50	AF077560
TUZX pm4.9	F-5' ATCTGACAGGGCCACCATAC 3' R-5' AGTCGAGTCTGAATAAGCG 3'	56	AF077568

Variation in microsatellite loci and departure from Hardy-Weinberg equilibrium

Number of alleles per locus (A), effective number of alleles (a_e), allelic size range in bp (R), frequency of the most common allele (F) and number of genotypes (G) were determined for each population at each locus to analyse the variation in microsatellite loci of three populations. The number of alleles per locus was calculated using GENEPOP Ver.3.4 (Raymond and Rousset, 1995). The effective number of alleles (a_e) was estimated using the formula: $a_e = 1/\Sigma Xi^2$, where X_i is the frequency of the ith allele at the locus. Allele frequencies for each population at each locus were calculated based on the number of alleles provided by GENEPOP. The frequency of each allele at each locus was obtained by dividing the total number of alleles in each population by the total number of alleles at the particular locus (Xu *et al.*, 2001). To test departures from Hardy-Weinberg equilibrium (HWE), the observed heterozygosity (H_o) was obtained using observed heterozygotes divided by total samples. The expected heterozygosity (H_e) was calculated using the formula: $H_e = 1 - \Sigma p^2_i$ (Nei, 1987) (where p_i is the ith allele frequency).

Genetic differentiation among populations

F_{ST} values (Nei, 1987) which measure the reduction in the average proportion of heterozygous genotypes among populations, were estimated to assess genetic differentiation. Procedure given by Nei (1978) was used to estimate Fst values using POPGENE. The genetic divergence among populations was determined by a probability test or Fisher exact test and log-likelihood (G) based exact test using GENEPOP described by Raymond and Rousset (1995), which estimates the probability of the allelic and genotypic distribution being identical between all pairs of populations. Genetic population relationships were estimated by constructing unweighted pair group method with arithmetic mean (UPGMA) tree based on Nei's standard genetic distance (Nei, 1978): Method UPGMA modified from NEIGHBOR procedure of PHYLIP ver.3.5. The bootstrap value for the UPGMA tree was calculated by considering 1000 replicates across loci.

Results and discussion

Genetic variability in microsatellite loci

The number of alleles per locus (A), effective number of allele (a_e), allele size range (R), frequency of the most common allele (F) and number of genotypes (G) for each microsatellite loci are given in Table 2. A total of 101 different alleles were found across the eight loci analysed in the study. The total number of alleles per locus across three populations ranged from 5 to 23. The number of alleles per locus is relatively less compared to the earlier micro satellite surveys done on *P. monodon*: 14 to 28 alleles at 2 loci (Tassanakajon *et al.,* 1998); between 19 and 30 alleles at 5 loci in

Table 2. Allelic variability at eight microsatellite loci in *P. monodon*

Parameters	Population	Locus							
		TUXZ Pm2.27	TUZX Pm2.28	TUZX Pm2.36	TUZX Pm 2.69	TUZX Pm4.84	TUZX Pm4.87	TUZX Pm6.1	TUZX Pm4.9
A	Kakinada	13	6	11	5	9	9	10	18
	Chennai	15	6	10	5	8	11	14	19
	Mumbai	11	6	9	5	8	8	15	17
	Total	16	6	13	5	10	11	17	23
a_e	Kakinada	9.19	3.83	7.19	3.23	5.69	5.63	4.44	11.63
	Chennai	10.17	3.83	5.49	4.03	5.21	7.15	6.17	12.50
	Mumbai	5.22	4.14	5.12	2.95	4.16	5.28	6.44	11.98
R	Kakinada	192 -222	194-214	156-181	257-280	303-321	331-353	135-151	199-339
	Chennai	192-222	194-214	163-183	257-280	303-317	331-353	137-169	195-340
	Mumbai	194-220	194-214	153-173	257-280	303-317	331-345	135-169	195-272
F	Kakinada	0.14	0.43	0.22	0.48	0.26	0.31	0.32	0.44
	Chennai	0.15	0.40	0.29	0.36	0.32	0.27	0.23	0.10
	Mumbai	0.26	0.31	0.34	0.50	0.42	0.28	0.25	0.065
G	Kakinada	42	51	51	46	50	51	53	96
	Chennai	46	50	52	45	54	52	52	98
	Mumbai	43	49	53	42	55	51	50	94

A : Number of allele per locus, a : effective number of alleles, R : allelic size range in bp, F : frequency of the most common allele, G : number of genotypes

Thailand (Supungul *et al.*, 2000); 34 and 84 alleles at 3 loci in Australia (Brooker *et al.*, 2000) and 6 to 54 alleles at 6 loci in Philippines (Xu *et al.,* 2001).

Minimum numbers of alleles (5) were observed in locus *Pm2.69* and the maximum numbers of alleles (19) were observed in locus *Pm4.9*. In addition, 5 to 13 null alleles were also observed among the populations at all loci. The effective number of alleles (a_e) ranged from 2.95 to 12.5 among the loci. Allele sizes ranged from 135 to 353 bp across the eight microsatellite loci. The number of genotypes in the loci for three populations ranged from 42 to 98 across the loci, the least number of genotypes (42) observed in locus *Pm2.69* (Mumbai) and the maximum (98) was observed in locus *Pm4.9* (Chennai). All the loci analysed in the study were highly polymorphic and this polymorphism signifies that there is considerable genetic variation present within the stock at the molecular level, which indicates a scope for genetic improvement of this species.

The measure of the amount of heterozygosity across the loci can be used as a general indicator of the amount of genetic variability. The observed heterozygosity (H_e) among the eight loci ranged from 0 to 0.168, in which the loci *Pm6.1* and *pm4.9* showed heterozygosity and others were homozygous (Table 3). The mean observed and expected heterozygosity was 0.042 and 0.835, respectively. The observed mean heterozygosity is less than the earlier observations such as 0.78 by Supungul *et al.* (2000); between 0.47 and 1.00 across six loci by Xu *et al.* (2001) and 0.643 to 0.753 across ten loci by Mandal *et al.* (2012) in *P. monodon*. In this study, we could observe heterozygous alleles only in locus *Pm6.1* and *pm4.9*, in spite of observing more number of genotypes as well as high number polymorphic loci in all populations. The low level of heterozygosity observed in this study leads to heterozygote deficits in all the populations at eight loci. Similar to our observations, Xu *et al.* (2001) has reported 5 cases of heterozygous deficit for 3 microsatellite loci in 4 populations of *P. monodon*. Several explanations for heterozygous deficit have been proposed including inbreeding, null allele, technical artifacts, population mixing, mixture of cohorts and others (Cruz *et al.*, 2002; Castric *et al.,* 2002). Considering the above statement, parameters such as population mixing between Kakinada and Chennai in the east coast and presence of null alleles (5-13 alleles observed in this study) might have caused the loss of heterozygosity in the three different populations of the species.

Genetic structure and isolation by distance

Geographic differentiation of *P. monodon* was first established in Australian population based on allozyme analysis (Benzie *et al.*, 1992). Soon after, microsatellites were used which revealed higher population substructure than allozyme in different species of shrimps (Brooker *et al.*, 2000; Supungul *et al.*, 2000). In the present study, the F-statistics was estimated for *P. monodon* populations across the eight microsatellite loci. The measure of inbreeding (F_{IS}) for *P. monodon* at eight microsatellite loci ranged from 0.795 to 1.00 (average: 0.949) (Table 4) and a positive value indicates the deficiency of heterozygotes in the stocks. The average measure of population subdivision (F_{ST}) for the populations at eight microsatellite loci is shown in Table. 4. The F_{ST} value has a theoretical range from 0 to 1, and higher F_{ST} value indicates more subdivisions between the populations in comparison. In the present study, we found the overall F_{ST} value as 0.021 across all the loci. According to Weir and Cockerham (1984), an F_{ST} value of less than 0.05 imply there is negligible genetic variation and more than 0.25 indicates great genetic variation among the species. Hence, the observations of the study indicate the presence of negligible genetic variation among three populations of *P. monodon* from Indian waters for eight particular microsatellite loci.

Earlier studies using microsatellite markers for stock characterisation in shrimp showed different level of genetic variability. Studies in *L. vannamei* by Jimenez *et al.* (2005) reported the calculated F_{ST} value as 0.055 and inferred significant level of genetic differentiation from Mexico to Panama population using five microsatellites markers. Sbordoni *et al.* (1986) reported a depression of genetic variability in cultured *Penaeus japonicus* and the

Table 3. Homozygosity and heterozygosity for three populations at eight microsatellite loci

Locus	loci								Mean
	TUXZ *Pm2.27*	*TUZX* *Pm2.28*	*TUZX* *Pm2.36*	*TUZX* *Pm2.69*	*TUZX* *Pm4.84*	*TUZX* *Pm4.87*	*TUZX* *Pm6.1*	*TUZX* *Pm4.9*	
Observed Ho	1.00	1.00	1.00	1.00	1.00	1.00	0.8333	0.8322	0.9582
Expected Ho	0.0989	0.2381	0.1552	0.2896	0.1939	0.1367	0.1539	0.0552	0.1652
Observed He	0.00	0.00	0.00	0.00	0.00	0.00	0.1667	0.1678	0.0418
Expected He	0.9011	0.7619	0.8448	0.7104	0.8061	0.8633	0.8461	0.9448	0.8348

Ho = Observed heterozygosity, He = Expected heterozygosity

Table 4. F-statistics of three *P. monodon* populations of India at eight microsatellite loci

Locus	TUXZ Pm2.27	TUZX Pm2.28	TUZX Pm2.36	TUZX Pm2.69	TUZX Pm4.84	TUZX Pm4.87	TUZX Pm6.1	TUZX Pm4.9	Mean
F_{IS}	1.00	1.00	1.00	1.00	1.00	1.00	0.795	0.8204	0.9487
F_{IT}	1.00	1.00	1.00	1.00	1.00	1.00	0.799	0.8248	0.949
F_{ST}	0.035	0.018	0.017	0.009	0.009	0.034	0.020	0.025	0.021

F: Fixation, I:Individuals, S: Subpopulation, T: Total population, F_{IS}: Hardy-Weinberg distribution of genotypes of individuals within subpopulation, F_{IT}: Distribution of individual genotypes within the total population, F_{ST}; Genetic differentiation of subpopulation within total population

cause was attributed to random drift, bottleneck effect, natural and artificial selection or stock transfer. Loss of genetic diversity in population studies on penaeid shrimps using allozymes showed little subdivision among these marine species (Mulley and Latter, 1980; Harris *et al.*, 1990), which was attributed to dispersal ability and life history of shrimps, combined with lack of physical barriers (Lester, 1979). Brooker *et al*. (2000) reported the population structure of *P. monodon* in Australian waters using three microsatellite loci, where they could not find genetic differentiation among the populations separated by 3000 km. However, genetic fragmentation was observed in *P. monodon* populations from the Philippines coast which were separated by 525 km using six microsatellite loci by Xu *et al*. (2001). Similarly, a strong and statistically significant genetic stock differentiation was also observed among tiger shrimp populations in India by Mandal *et al*. (2012). The negligible genetic variation observed in the present study is concordant with the observations of Sbordoni *et al*. (1986) and Brooker *et al*. (2000). With the help of using information of earlier studies and knowledge on geographical area and fishing activities of sampling location, a conclusion could be made for cause of loss in great genetic differentiation among the populations of *P. monodon*. Hence, we postulate that the following factors have caused the loss of genetic diversity among three populations : high-level of gene flow during planktonic larval stage of *P. monodon* in the east coast between Kakinada and Chennai, as well as shrimp trawling in both the coasts contributing to genetic drift leading to loss of heterozygosity. Overfishing and deterioration of the breeding environment resulted in less recruitment and reduced genetic variability. In addition, a smaller sampling size used in the study might have also affected the calculated values. Although less genetic differentiation was observed among the studied populations, the genetic relation inferred from the probability value of pair-wise allelic differentiation (Table 5), and Nei's genetic distance estimation explained by bootstrapped neighbor-joining tree (Fig. 2) showed that the Kakinada and Chennai populations are closely related than Kakinada and Mumbai, as well as Chennai and Mumbai populations.

Table 5. p-values for pairwise comparison among different populations of *P. monodon* (Raymond and Rousset, 1995)

Population pair	p value	
	Genic differentiation	Genotypic differentiation
Mumbai and Chennai	0	0.004
Mumbai and Kakinada	0	0.0004
Chennai and Kakinada	0.0006	0.2632

p<0.01

Genic differentiations are concerned with the allelic distribution of alleles in the various populations. The null hypothesis tested is, Ho: "the allelic distribution is identical across populations

Genotypic differentiations are concerned with the distribution of genotypes in the various populations. The null hypothesis tested is, Ho: "the genotypic distribution is identical across populations
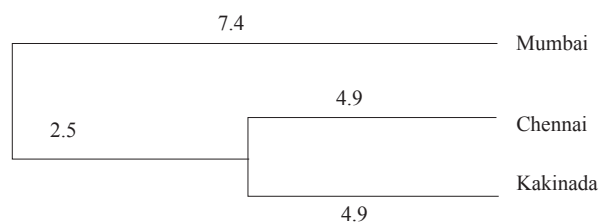

Fig. 2. UPGMA dendrogram showing the phylogenetic relationship among three populations of *P. mondon*. The scale represents Nei's (1978) genetic distance.

The methods used in the study revealed low degrees of population differentiation in the species but based on pair-wise allelic differentiation and Nei's genetic distance, the three populations of *P. monodon* could be allocated in to two different populations, east coast and west coast populations. However, presence of polymorphic loci indicates there is considerable genetic variation present within the stock at a molecular level, which indicates scope for genetic improvement of this species. Eventually, a better conclusion on the genetic variability cannot be drawn based on eight loci used in microsatellite analysis. Further, studies with specimens from a broader geographic area and more loci for analysis are required for better understanding of the genetic structure of *P. monodon*. The basic information obtained in this study on the genetic variability of *P. monodon* could be important for fishery, aquaculture management and conservation of the species.

Acknowledgements

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