Phylogeny and genetic variation within population of *Tachypleus gigas* (Müller, 1785)

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Isolated population of Indian horseshoe crabs, *Tachypleus gigas* plays an important role in the ecology of several marine organisms, and is being scrutinized for its abundance and morphology by many researchers. However, limited information is available about its genetic variability and evolution. Samples of horseshoe crab were collected from the east coasts of India and analysed for their phylogenetic relationship, genetic variability and structure within population based on the cytochrome oxidase I (*COI*) gene sequence. Analysis of molecular variance revealed two groups with significant genetic differentiation indices (F_{ST} = 0.544, $p < 0.001$), and the number of migrants (*Nm*) was estimated as 0.11 individuals per generation. Maximum likelihood results revealed two distinct clusters, showing that the evolution of the Indian population was genetically diverse forming a separate clade from other Southeast Asian populations, moderately with a low gene flow. Considering the ecological, economic and evolutionary significance of *T. gigas* and its declining population, there is a pressing need for conservation measures.

Keywords: *COI* gene, genetic variation, horseshoe crab, phylogeny.

HORSESHOE crabs play an integral role in sustaining ecological food web for migrating shorebirds, finfish, including loggerhead turtles[1]. They are a unique group of animals in maintaining their genotype almost unchanged for millions of years[2,3]. They are marine chelicerate arthropods under the class Merostomata, a sister group to scorpions and ticks followed by spiders and crabs[2]. Four extant species, *Tachypleus tridentatus*, *Tachypleus gigas*, *Carcinoscorpius rotundicauda* and *Limulus polyphemus* were distributed worldwide[4]. Based on the global distribution, the Atlantic horseshoe crab (*L. polyphemus*) is commonly found in the Gulf of Mexico, and *T. gigas* is predominantly found along the east coast of India (Odisha), Indo-China, North Vietnam, Borneo and Celepes. *T. tridentatus* occurs from the Northern shores of Japan to South Vietnam and along the western islands of the Philippines. Mangrove horseshoe crab *C. rotundicauda* is found from the northern shores of the Bay of Bengal to the southern coast of the Philippines. *C. rotundicauda* and *T. gigas* inhabit the coastal waters of the Bay of Bengal (India) and the continental shelf region within 48 km up to 312 km (refs 5, 6). However, they exhibit restricted distribution in the west coast of India.

Molecular studies have widely been used to determine generic variation as well as predicting their phylogeny of population structure[7]. The highly conserved region of Mitochondrial Cytochrome Oxidase subunit 1 (*mtCOI*) gene is considered as a powerful marker at the lower level of species due to high mutation rates making population subdivisions[8]. Hence highly variable region of *mtDNA* gene is useful for phylogenetic studies and genetic variation[9]. The genetic integrity between the northern and southern populations of American horseshoe crabs (*L. polyphemus*) was observed along the Florida coast using *mtDNA* gene. High turbidity and strong currents would appear to make a potential barrier to movement[10]. Similarly, genetic variation within the population of *T. tridentatus* was found in East Malaysia[11,12]. The aim of this study was to determine the phylogenetic relationship and genetic variation within the *T. gigas* population using *mtCOI* gene along the east coast of India.

Materials and methods

Sample collection

Three samples were collected from the east coast of India (Chandipur, Baleshwar, Odisha) during 2008 (Figure 1). Specimens were identified, weighed, sexed and morphometric characteristics were noted[13]. A part of walking leg was removed from horseshoe crabs using sterilized scissors and the internal soft tissue was preserved in 95% ethanol. All horseshoe crabs were released back to the beach in live condition to ensure their sustainability[14].

Laboratory procedure

The genomic DNA was extracted from muscle tissues using Qiagen DNA Easy Blood and Tissue kit according to the manufacturer's instruction. DNA concentration was quantified spectrophotometrically using NanoDrop (Thermo Scientific, DE, USA) and the partial *COI* gene sequence was amplified by PCR using universal primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′)

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and HCO2198 (5′-GTCTAACCGCGGTAGCTGGCAC-3′)[15]. The amplification reaction was carried out in a 50 µl containing ~30 ng genomic DNA, 0.5 U Sigma *Taq* DNA polymerase, 1 × *Taq* buffer, 2.5 mM $MgCl_2$, 400 µM of each deoxynucleotide triphosphate (dNTP) and 0.4 µM of forward and reverse primers. PCR reactions were performed on a ABI thermal cycler with an initial denaturing step at 94°C for 2 min, 39 amplification cycles (94°C for 30 sec, 50°C for 60 sec, 72°C for 90 sec), and a final elongation step at 72°C for 5 min, followed by a 4°C, and the amplified DNA was separated through electrophoresis in 1% agarose gel and purified (QIAGEN PCR Purification kit, Qiagen, USA). Final DNA product was sequenced (ABI 3130xl sequencer) and the obtained chromatogram was edited using ABI sequence scanner software 1.0v. The sequences were analysed using BLAST to find similar sequences in the NCBI database; finally identified sequences were deposited in NCBI GenBank (accession numbers KJ825847–KJ825849) and other DNA sequences were taken from the NCBI website (Table 1).

The *mtDNA* gene sequences were edited using BioEdit version 7.0.1 (ref. 16) and aligned with CLUSTAL-W using MEGA6 (Molecular Evolutionary Genetics Analysis) software[17]. Genetic variation of *T. gigas* was calculated using Arlequin 3.0v for analysis of molecular variance (AMOVA) with 1000 permutations as implemented[18]. Phylogenetic relationship within populations was inferred using maximum likelihood (ML; 2000 bootstrap replicates) approaches to construct in a MEGA6 phylogram[17]. *L. polyphemus* was used as the outgroup, and best tree was selected and imported into Tree view to produce a 50% consensus tree with ML support values added to the tree nodes. Unique haplotypes (h), nucleotide diversity (π)[19] and pairwise F-statistics (F_{ST}) were calculated as genetic distances based on pairwise differences between populations. The demographic history of *T. gigas* was inferred by mismatch distribution analyses using DnaSP software 4.50.3v (ref. 20). Gene flow was estimated based on the equation $Nm = 0.5 \times [(1/F_{ST}) - 1]$, where N is the effective number of females and m is the migration rate by GENALEX 6 (ref. 21). Percentage of AT and GC content was calculated using BioEdit software[16].

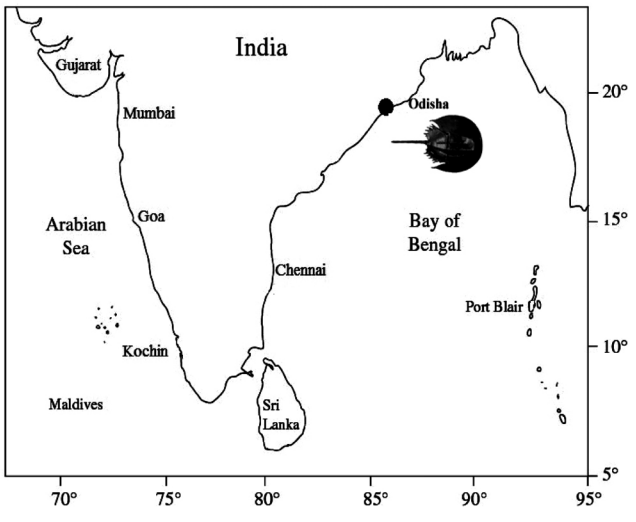

Figure 1. Map showing the location of sample collection.

Results and discussion

Phylogenetic analysis

Phylogenetic analysis of *T. gigas* from three different regions (Southeast Asia, Malaysia and Thailand) resulted into one cluster while Indian samples formed a separate cluster (Figure 2). Phylogenetic analysis showed two clades within the population and geographic structure, suggesting migration between historically isolated populations. Phylogeny represents a topology where the two form a separate branch from *T. gigas*. In India, the population of *T. gigas* was significantly better than Southeast Asia ($P < 0.05$), indicating that the mtDNA lineage is certainly divergent from the two bristle row lineage. The numbers at each node in Figure 2 represent percentage of bootstrap values based on 2000 pseudo replications of ML analyses. *T. tridentatus*, *C. rotundicauda* and *L. polyphemus* used as an outgroup were clearly clustered in a separate branch to prove the reliability of the constructed phylogram. The reconstructed phylogenetic tree clearly indicates that Indian horseshoe crabs are related to the Southeast Asian population. The *L. polyphemus* species from the east coast of North America has

Table 1. Detailed information of the sampling location

Species	Origin	*COI* gene sequence
India		
	Odisha	KJ825847
		KJ825848
		KJ825849
Southeast Asian		
Tachypleus gigas	Malaysia	JF896114
		JF896108
		JF896107
	Thailand	HQ588748
		HQ588749
	Malaysia	JF896111
		JF896109
Tachypleus tridentatus		JN018216
	Thailand	HQ588752
Carcinoscorpius rotundicauda		AF370828
	Malaysia	JF896106
		JF896105
	Thailand	HQ588754
		HQ588746
		HQ588750
Limulus polyphemus	Atlantic	HQ588751
		EU834780

Table 2. Analysis of molecular variance for *COI* gene within *T. gigas* populations

Source of variation	Degree of freedom	Sum of squares	Contribution of variation (%)	Percentage of variation	F_{ST}	P
Among populations	3	442.143	31.708	54.45	0.544	0.001
Within populations	20	623.200	31.160	45.55		
Total	23	1177.000	68.402	100		

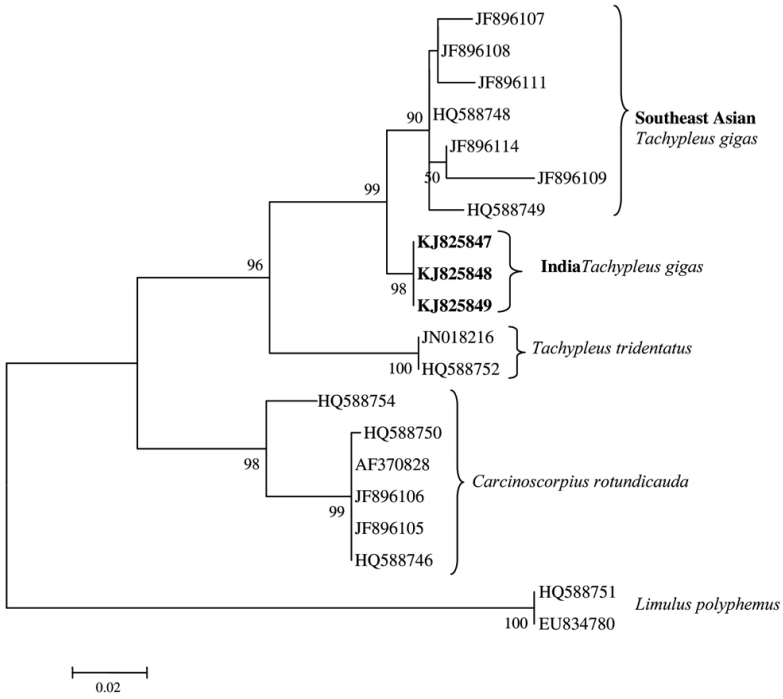

Figure 2. Phylogenetic tree showing the relationship between Indian and Southeast Asian *Tachypleus gigas*. (Inference based on *COI* gene and bootstrap values >50% are shown in the nodes. Scale bar 0.02 nucleotide substitutions per nucleotide positions.)

genetically isolated population differentiated from the three other species[22]. Another interesting point emerged from the phylogenetic relationship is that *T. gigas* is closer to *T. tridentatus* followed by *C. rotundicauda*. Xia[23] reported that partial mtDNA sequence analysis show high similarity index between *T. gigas* and *T. tridentatus*.

Gene flow and genetic differentiation

The fixation index (F_{ST}) was estimated to be 0.635 between the Indian and Southeast Asian populations. This indicates isolated and low gene flow between populations. It also implies lower migratory rate per generation (*Nm*: 0.12) between the Indian and Southeast Asian samples. The limited migration pattern of horseshoe crab population clearly proved that a geographical barrier to gene flow was highly restricted between populations. However, AMOVA within population yielded $F_{ST} = 0.544$, ($P < 0.001$), indicating a significant level of genetic variation among populations (Table 2). In fact, adults of *T. tridentatus* stay in the deeper waters and migrate to shallow areas for reproduction[5]. The trilobite larvae settle right after hatching and juveniles spend their life stages at or near the natal beach for feeding[24]. These characteristics indicate that larvae and juveniles have a limited migratory pattern, but adults can travel long distances by swimming with the sea current. The geographical barriers may be an important factor limiting the gene flow. For instance, the small geographic range in the Bay of Bengal was the fundamental cause of population subdivision, that is semi-closed, and as a consequence the population might have limited genetic exchange with those outside the Bay[25]. Similarly, the genetic break of *L. polyphemus* species was also observed between the Gulf of Mexico and Atlantic populations which suggest that Florida Peninsula might be the barrier to gene flow[26].

Genetic and haplotype diversity

The trimmed mtCOI dataset consists of 650 bp and three individuals from localities on the east coast of India (Odisha). Of those, three mtDNA sequences were taken

Table 3. Variation in the composition of nucleotides

Populations	*T. gigas*		*T. tridentatus*	*C. rotundicauda*	*L. polyphemus*
	India	Southeast Asia			
Length (bp)	653	538	588	569	633
A%	27.81	27.69	29.35	30.32	28.59
C%	21.84	21.77	18.71	20.63	23.7
G%	15.97	15.85	15.63	15.81	16.9
T%	34.39	34.69	36.32	33.2	30.81
G + C content (%)	37.81	37.62	34.33	36.44	40.6
A + T content (%)	62.19	62.38	65.67	63.53	59.4

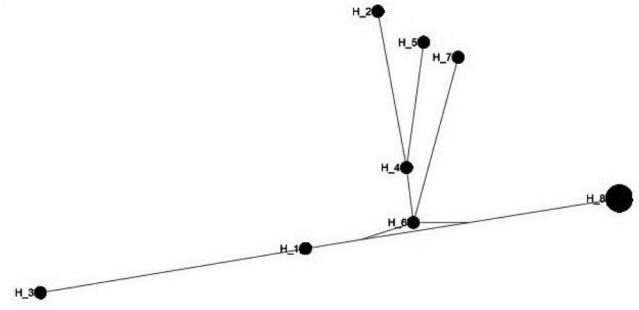

Figure 3. Median-joining network showing the phylogenetic relationships among partial mtDNA *COI* gene haplotypes of *T. gigas*. Numbers crossing lines characterize sites of nucleotide substitutions; circled areas show the proportion of haplotypes.

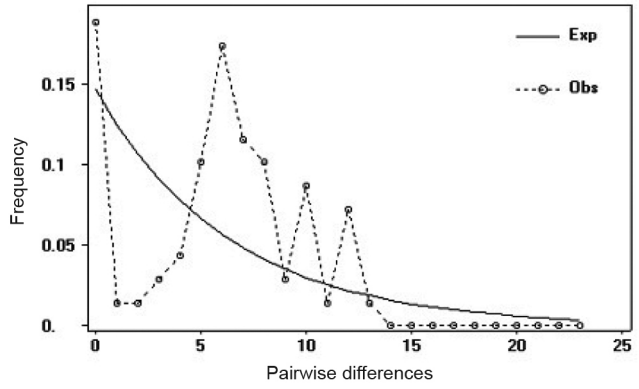

Figure 4. Mismatch analyses of eight haplotypes of *T. gigas*.

for average nucleotide base composition and adenine–thymine bias (T for 34%, A for 28%, C for 22% and G for 16%). AT content (62%) was much higher than that of GC (38%) (Table 3). Eight haplotypes (Figure 3) (designated as H1–H8) were defined from all partial mtCOI sequences. Intra-population genetic diversity varied among populations with haplotype diversity (h) being 0.019, whereas nucleotide diversity (π) was 0.88 and showed the high genetic diversity. Among the diverse eight haplotypes, H8 was observed as dominant type in the Indian locality, and H6 and H7 were shared by Thailand. H4 was observed in Malaysia, and others sample-specific haplotypes. Five haplotypes (H1, H2, H4, H5 and H6) were one mutational step away from H3, which was most likely an ancestral type. H2 and H4 were also one mutational step away from H1. The prolonged star-like network indicated the stable existence of the historic population of horseshoe crab. Figure 3 also shows the topology of the median-joining network of eight haplotypes. Figure 4 shows the mismatch frequency spectra for the eight populations.

The genetic variation in horseshoe crab indicated that expansion of refugial populations occurred in less genetically diverse species living in the recently colonized population[7,27]. Similar observations were made in other aquatic organisms such as bivales[27,28]. The geographically isolated population showed a restricted gene flow and high genetic variations between the populations[8,29,30]. The nucleotide diversity of *T. tridentatus* populations from the closer geographical area of Taiwan coast showed similar results to the restricted gene flow within the population[25]. The loss of suitable spawning and feeding grounds was due to coastal development and population explosion[31]. Furthermore, the appropriate protected areas could be planned to conserve the horseshoe crab and its habitats.

Conclusion

The phylogenetic analysis of *mtDNA* gene sequence showed that it clustered with sister species in the individual group, proving evolution of the species. Reconstruction of phylogenetic tree and genetic variation data of *T. gigas* species clearly proved that the Indian and Southeast Asian populations are more genetically related to *T. tridentatus* other than horseshoe crab species. Interestingly, restricted gene flow was observed between India and Southeast Asian populations. However, the distribution of Indian population was geographically isolated. Therefore, the high genetic variation indicated that measures need to be taken to protect the rare and threatened marine species. The available genetic information on Indian horseshoe crab could be used in different conservation strategies for their sustainable fishery management along the east coast of India (Odisha). This study also suggests the need for further monitoring genetic changes within the population of Indian horseshoe crab.

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