Assessment of Genetic Variation in COI Gene of Horseshoe Crab (*Tachypleus Gigas*) Population Along the Coast of the Bay of Bengal in Odisha, India

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Abstract

Horseshoe crab (*Tachypleus gigas*) is an archaic group of marine creature which plays a vital role in the saline ecosystem. Many researchers emphasize and enhance the knowledge about the horseshoe crab's basic biology, morphology, and ecology, whereas very little information is available about its population genetics. We attempted to develop a baseline database about the ecology, phylogeography, and genetic variation among the horseshoe crab population from Odisha, India. We collected 152 samples of horseshoe crab from the coastal area of the Bay of Bengal. The generated Cytochrome C Oxidase Subunit I gene (COI) sequences of *T. gigas* were compared with the sequences of *T. gigas* obtained from GenBank. The GenBank sequences were of two populations from South China and Malaysia. A total of 26 unique haplotypes were observed in three populations of *T. gigas*. Pairwise F-statistic distance (F_{ST}) between South China-India was 0.708; Malaysia-India was 0.608, and South China-Malaysia was 0.136. It indicated that the South China population was closely related to the Malaysian population and the Indian population was appeared to be genetically distinct from the other two populations. It signifies the ecological importance of the Indian population. Furthermore, the migrant per generation (Nm) was 0.16, which indicated a low gene flow among *T. gigas* populations. The haplotype diversity (Hd) and nucleotide diversity (π) were 0.58826 and 0.00476, respectively. This study would help lay future strategy and conservation of horseshoe crab across the Bay of Bengal.

Introduction

Horseshoe crabs are one of the archaic groups of marine organisms with significant economic value and scientific significance. These animals play an important role in both marine and estuarine ecosystems. Four existing species of horseshoe crabs are reported throughout the world. Of these, *Limulus polyphemus* [1] is found on North America Atlantic coast, whereas other three species of horseshoe crabs like *Tachypleus gigas* [2], *Tachypleus tridentatus* [3] and *Carcinoscorpius rotundicauda* [4] are found in the Asian countries ranging from 6°S to 31°N and between 90°E and 118°E [5]. Out of the four horseshoe crab species, *Tachypleus gigas* and *Carcinoscorpius rotundicauda* have been reported with overlapping distribution in many regions in the Bay of Bengal, India and South China Sea [6,7]. *Tachypleus gigas* prefer sandy areas and *C. rotundicauda* is found in muddy regions [8-12]. However, trispine Asian horseshoe crab *Tachypleus tridentatus* have a preference to occur in the coastal zone of shallow water in southern China ranges from Vietnam to Japan. In the Indian sub-continent, the maximum numbers of horseshoe crabs are distributed along the Bay of Bengal coast of India, ranging from Sundarban mangrove forest of West Bengal state to Dhamara of Odisha state [13,14]. In Odisha state, the coastal region of the Balasore district is richly populated with horseshoe crabs. Horseshoe crab inhabits three regions like the coastal bay, intertidal mud zone or sand flat zone, and deepwater regions [15]. Balasore has qualified as a preferable place for breeding due to the intertidal zone, which helps for their spawning activity [10]. It has been reported that the *T. gigas* and *C. rotundicauda* come to the estuary for spawning purposes in pairs during the tidal movement of spring seasons in India [16,17]. The population of horseshoe crabs is declining rapidly due to overfishing, water pollution, habitat destruction, resulting in reduced abundance and quality of prey available as a food source to horseshoe crab [6]. Hence, it is well accepted and considered an endangered marine organism in India and other regions [18-20]. Several controversies have been raised among the evolutionary biologist about the horseshoe crab phylogeny from the last several years. They are often considered the foremost example of long-term survival in marine. These marine organisms still retain their body morphology and stable genetic structure over millions of years [21-23], hence called as 'Living fossil' [24], 'Phylogenetic relict' [25] and becoming endangered marine animals. Generally, in the marine ecosystem, organisms exhibit strong gene flow for their dissemination activity across the spacious geographic ranges [26]. Some habitually display a low level of genetic differentiation for their incapability of long-distance dispersal over the large geographic scale [27]. Horseshoe crabs are lean migratory animals that are probable to have subdivisions population on a small geographic scale [26, 28]. They reveal a low level of genetic diversity throughout their entire evolutionary history. The allozyme [25,29], restriction fragment length polymorphism (RFLP) of mtDNA [30], and microsatellite analysis [31] revealed that the population of American horseshoe crabs (*Limulus polyphemus*) was genetically distinct in the Gulf of Mexico and the Atlantic coast. In a small geographic range of North America, limited gene flow was observed through COI gene analysis [28]. Two genetic groups were detected based on the distribution of haplotypes and the significant genetic structure of Japanese horseshoe crabs (*T. tridentatus*) populations by the mtDNA AT-rich region [32]. A few research studies have been conducted to explore the phylogeny and phylogeography of four extant species using various molecular methods. However, very little knowledge is available about the horseshoe crab's population genetics diversity along the Bay of Bengal coast in India. A molecular study is a powerful tool for investigating genetic differentiation and it has widely been used to determine population genetics, phylogeography and genetic structure. It possesses a rapid rate of evolution, lack of recombination, and maternal inheritance [33]. Mitochondrial Cytochrome C Oxidase subunit I (COI) is often considered a conserved region and used as a robust marker for the invertebrates and marine species due to a high degree of polymorphism leading to population subdivisions (Hu et al. 2009)[34]. The objective of this study was to examine molecular variations, genetic diversity and historical demography of Indian horseshoe crab in the select area of the Bay of Bengal coast.

Materials And Methodology

2.1 Sample collection and DNA extraction

We collected 152 tissue samples from the dead remains of horseshoe crabs from fourteen sampling sites of the coastal areas of the Bay of Bengal (Table 1). Samples were stored at -20 °C in 70% ethanol for molecular analysis. Genomic DNA was extracted from the tissue sample using the standard Proteinase K digestion/phenol-chloroform method [35], and a standardized protocol was adopted to extract DNA [36]. The extracted DNA was quantified in a QIAxpert spectrophotometer. Isolated DNAs were diluted in a final concentration of 40-50 ng/μl for PCR amplification and stored at 4°C.

2.2 PCR amplification and sequencing

We used conserved primers for PCR amplification and sequencing of the mtDNA COI gene [37]. The total 20 μl reaction volumes were prepared for PCR reactions using 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, and 50 mM KCl), 1.5 mM MgCl₂, 1 × BSA, 0.2 mM of each dNTPs, 3 pmol of each primer, 0.5 units of

DreamTaq DNA Polymerase (Thermo Fisher) and 1 µl (~ 30 ng) of template DNA. The PCR conditions were initial denaturation at 95 °C for 5 minutes and 95°C for the 40s, annealing temperature at 45°C for 50s, 72°C for 50s followed by 5 cycles and second denaturation at 95 °C for 40 s, annealing at 51 °C for 50 s and extension at 72 °C for 50 s. The final extension was at 72 °C for 10 min, followed by 35 cycles, and maintained at 4°C until further use. For avoiding any cross-contamination, efficiency, and reliability, control and the negative reaction were taken during DNA extraction and PCR reactions. The PCR amplification was confirmed by electrophoresis on 2% Agarose gel stained with safe stain dye (0.5 mg/ml) and visualized under a UV transilluminator. The exonuclease-I and shrimp alkaline phosphatase (Thermo Scientific) was treated at 37°C for 20 min, followed by 85 °C for 15 min with the amplified PCR product to obtain intact DNA. The sequencing was performed from forward and reverse direction using BigDye v3.1 Kit in 3500 XL Genetic Analyzer (Applied Biosystems).

2.3 Data alignment and molecular analysis

The obtained DNA sequences were scrutinized for similarity and retrieved through BLAST sequences accessible from NCBI GenBank. Later the sequences were aligned by using Clustal X available in the BioEdit packages [38]. The IUPAC nucleotide was clean in BioEdit software. The numbers of variable sites and population diversity were analyzed using DNASP. A percentage similarity matrix and pairwise distance matrix were generated using MEGA X [39]. Evolutionary analyses were conducted in MEGA X. The haplotype median-joining network was generated by Network v.5.0.0.0 as well as PopArt software. In ARLEQUIN v3.11 software analysis of molecular variance (AMOVA), Tajima's *D* parameter estimations, gene flow, and mismatch distributions were estimated. Neighbour Joining, Maximum Parsimony and Maximum Likelihood tree were constructed based on 581 bp of the mitochondrial DNA COI/gene from *T. gigas*, and three out-group sequences of horseshoe crabs were derived from GenBank. Two different methods, maximum likelihood (ML) and Bayesian inference (BI) were adopted for phylogenetic analysis. In RAXML-VI v.7.0.0. Software analysis ML tree estimation was performed [40]. A Bayesian phylogenetic tree was generated in the beast. Based on the average haplotype diversity, genetic diversity (π) was estimated. Population pairwise nucleotide differences and their statistical significance were checked using Arlequin v.3.5.1.2 with 10,000 permutations. Geographical distribution and genetic differentiation of the haplotype population within the species were calculated by using PopArt software.

2.4 Divergence time estimation

The updated version BEAST v.1.7.5. was used for estimation of divergence among the horseshoe crabs species [41]. The single COI/gene sequence of each species is considered for analysis. We took *T. gigas* from the Bay of Bengal coast and another from the South China Sea, expecting a genetic gap in both the population for the large geographic distance. The remaining three species, namely *L. polyphemus* was from Mexico, *T. tridentatus* from the China Sea, *C. rotundicauda* from South Korea, for divergence estimates. The best model HKY was selected for this analysis from the JModel test and the base frequencies were not considered for estimation. We also took equal to empirical values. It was expected to evolve a phylogenetic tree with Yule speciation tree priors and a log-normal uncorrelated tranquil clock [42]. For all these analyses, 25 million generations made for a run. With the help of Tracer v.1.5, the Estimated Sample size (ESS-500) was checked and calculate the value in between the 200-600 ranges of all parameters [40]. We calibrated our tree with the divergence times described by Obst et al. (2012) [43]. According to Fisher [21] the divergence years of Limulidae-Tachypleidae were approximately about 150-130 Mya. The tree was calibrated with the value 140 Mya with a Standard deviation of 10 Mya.

Result

3.1 Molecular genetic diversity

We obtained 581 bp COI/gene sequence from 161 biological samples, of these 319 sites were constant and 34 were variable. We detected 26 distinct COI/mtDNA haplotypes with haplotype diversity (H_d) 0.6614, which occupied 5.852% of all analyzed sites, including 20 singletons and 14 parsimoniously informative sites (GenBank accession numbers MZ262422-MZ262447). It was also observed that there were 25 transitions, 13 transversions, or inversions/deletions. The estimated Transition/Transversion bias (R) was 1.94. The frequencies of nucleotide were A = 9.17%, T = 42.92%, C = 24.56%, and G = 23.36%. For estimating ML values, a tree topology was automatically computed. The maximum Log-likelihood for this computation was -746.630. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). Total 161 individual sequences were categorized into three populations as an Indian, Malaysian, and South China of *T. gigas*. Pairwise F-statistic distance (F_{ST}) between South China-India was 0.708; Malaysia-India was 0.608, and South China-Malaysia was 0.136. We also confirmed the intraspecies genetic distance, ranging from 0.00 to 0.05, while inter-species distance varied from 0.06 to 0.08 for the species under study. The highest distance was observed between *T. gigas* and *L. polyphemus*.

3.2 Genetic variation and haplotype diversity

To estimate the diversity indices, we used only the COI gene for molecular analysis. We detected two major haplotypes (H2 and H3) distributed in all sampling sites, while some local haplotypes were specific to the particular sites. Molecular diversity index such as haplotype (h) and nucleotide (π) diversity was estimated among the horseshoe crab's population in the Bay of Bengal. Nucleotide (π) and haplotype (h) diversity were found 0.00 to 0.00059 and 0.00 to 0.534, respectively. Overall nucleotide diversity and haplotype diversity in the Indian horseshoe crab population in the Bay of Bengal was 0.00265 and 0.578, respectively. We compared mtDNA genetic variation in the Indian horseshoe crab population in the Bay of Bengal with other *Tachypleus gigas* (South Chinese and Malaysian) populations across its distribution range. We found that Indian horseshoe crab has a comparatively lower level of genetic variation ($h=4$, $s=3$, mean pairwise difference = 1.53) than the well-connected populations of South China and Malaysia.

3.3 Historical demography

The selective neutrality test was taken to confirm population expansion Fu and Li's, Tajima's D , and Fu's statistics. The whole data set was included for this analysis. This analysis reveals that Tajima's D was -2.27032 ($P < 0.01$), Fu and Li's was -4.736415 ($P < 0.02$), while Fu's F_s value was -22.25092 ($P < 0.05$). Our data suggest that the *T. gigas* has experienced a sudden demographic expansion. In the mismatch distribution, a smooth and unimodal curve (Fig. 2) was observed among the fourteen geographic populations. The analysis of mismatch distribution was consistent with Fu's F_s statistical test for the population expansion model.

3.4 Phylogenetic analysis

Based on the partial sequence mtDNA COI gene, the phylogenetic tree was established. The best model was also considered as GTR+I from the JModel test. Only *T. gigas* species were included in the time of phylogenetic analysis. 17 unique haplotypes were obtained from 152 samples. These 17 haplotypes and ten sequences of *T. gigas* from NCBI GenBank were included for phylogenetic analysis. Interestingly our sample data categorized into two major subpopulations. 11 haplotypes fall under one subpopulation and the remaining six were in another group. In contrast, these were believed a sister group relationship with each other. Each phylogenetic partition was significantly different from one another ($P < 0.01$). Its overall analysis yielded constant phylogeny. In our study, open and highly supported the connection between *Tachypleus gigas* and *Tachypleus tridentatus* was observed than the *C. rotundicauda*, which showed sister taxon relationship, i.e., monophyletic genus *Tachypleus*. It was also strongly supported for *Tachypleinae* [44] between *Tachypleus* and *C. rotundicauda* to establishing phylogenetic clade as a monophyletic relationship.

3.5. Divergence time estimates

We obtained 150-130 Mya divergence between *Limulidae-Tachypleinae*. The diversification of Asian horseshoe crab *Tachypleinae* occurred during 50 Mya (22–90 Mya), and *Tachypleus* genus was originated in 36 Mya (14–65), while the internal split of the *T. gigas* across the South China-Indian was dated to 6 Mya (2–15), shown in Fig. 2.

Discussion

The marine biodiversity crisis has increased due to heavy anthropogenic activity and resulted in the depletion of habitats for marine organisms. Some of them have got extinct without our knowledge or ever having registered their existence. To assess the divergence time, genetic differentiation, phylogeographic pattern, and genetic structure of the horseshoe crab populations, we used the mtDNA gene (COI) for molecular analysis. Our finding was congruent with a previous study [45], which displayed the morpho-static structure of horseshoe crabs due to the lack of variation in molecular genetics. Horseshoe crab species showed a slow divergence rate compared to other arthropods [46] due to their long generation time and sexual maturity after ten years [47]. We included three major *T. gigas* populations such as India, South China and Malaysia. The South China population was closely related to the Malaysian population, and the Indian population was appeared to be genetically distinct from the other two. It can be explained due to its least dispersal capability in the marine ecosystem [26]. We confirmed the intraspecies genetic distance ranged from 0.00 to 0.05 while inter-species distance varied from 0.06 to 0.08. The highest genetic distance was observed between *T. gigas* and *L. polyphemus*. The *L. polyphemus* was restricted in the Gulf of America and the Atlantic coast only. It showed different genetic appearances. Due to this characteristic, the geographical barrier played an important role in dividing the genetic break for which they may not participate in genetic exchange and the species. The changes in sea level are the major factors to acclimatize/adapted the species to the particular environment resulting in genetic diversity. Hence, we can assume that they are pretty far from the genetic distinct of this species. We also conclude that the Indian horseshoe crab has a comparatively lower level of genetic variation ($h = 4$, $s = 3$, mean pairwise difference = 1.53) than the well-connected populations of South China and Malaysia. But it was quite similar to the geographically distinct populations. Therefore, it might also be possible that the Indian horseshoe crab population's isolation due to habitat subdivision was the probable reason for lower genetic variations. It suggested significant population genetic differentiation throughout the range of *T. gigas* along the coast of the Bay of Bengal. Among the 26 haplotypes, the major two H2 and H3 dominating subpopulations were observed on the Bengal coast. The haplotypes H13, H26, H27, H16 and H21 were one mutation away from the H2 haplotype and the rest H18, H22, H17, H20, H19, H23, H25, H15 and H24 were also one mutation away of H3 haplotype that means other haplotypes are just ancestor of both haplotypes. This analysis revealed that Tajima's D was -2.27032 ($P < 0.01$), Fu and Li's was -4.736415 ($P < 0.02$) while Fu's F_s value was -22.25092 ($P < 0.05$). Our data suggested that the *T. gigas* has experienced a sudden demographic expansion. In the mismatch distribution, a smooth and unimodal curve was observed among the 14 geographic populations. About 65 – 23 Mya during the Paleogene era, the Asian horseshoe crabs were diversified and split. Our results strongly supported the previous studies in which it was mentioned that the American species *L. polyphemus* was the sister taxon of three Asian species [21, 45, 48] and the Asian species was diversified around 55 Mya. As a first Asian species, *C. rotundicauda* (South Korean) was split approximately 50 (80 – 20) Mya. *T. gigas* showed a closer genetic distance with *T. tridentatus* than the *C. rotundicauda*. The Indian and South China species (*T. gigas*) divergence occurs during 15-2 Mya. *T. tridentatus* has diverged in around 65 – 15 Mya. Although, *C. rotundicauda* has a contrasting pattern of connectivity with *T. gigas*, which can reproduce its isolated lineage. The diversification of Asian horseshoe crab occurred during the Paleocene era (65 – 23 Mya). We confirmed two distinct horseshoe crab (*T. gigas*) subpopulations along the Bay of Bengal Bay in India.

The molecular analysis and evolutionary relationship among the Indian horseshoe crab (*T. gigas*) species significantly displayed a more distinct population. Additionally, in the phylogenetic tree, the genetic relationship of *Tachypleus gigas* is extreme nearer to *Tachypleus tridentatus* other than the *Carcinoscorpius rotundicauda* species. Within the *T. gigas* species of India and Southeast Asian populations, a restricted gene flow was observed. Interestingly, in the horseshoe crab population of India and the Southeast Asian population, a limited gene flow was observed. However, more detail and further scientific analysis are required to investigate and establish the genetic structure and the evolutionary history of the Indian Horseshoe crab population. An advanced molecular technique should be adapted to conserve valuable marine organisms. The Indian horseshoe crab was geographically distributed in a limited area on the Bay of Bengal coast, but now the current status is under severe exhaustion across all over its range even species is

vanishing in many estuaries. Due to anthropogenic activities, seawater pollution, overfishing and coastal development caused the loss of habitat vegetation. Accordingly, suitable conservation strategies should be adopted to protect the species and its habitats from extinction.

Declarations

Acknowledgment

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Author Contributions

BPD and SKG acquired resources and permission to collect biological samples and developed the concept and designed the framework. ST collected the biological samples and carried out sequencing. ST and SKG performed data analysis and wrote the paper. BPD and SKG finalized the manuscript. All the authors approved the final version of the manuscript

Disclosure statement

Competing Interests: The authors declare no competing interests

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Availability of data and material

The sequence data is submitted to GenBank under Accession No. MZ262422- MZ262447.

Ethical statement: The study was conducted using a tissue collected from the naturally dead animal and therefore, no Institutional Animal Ethics Committee approval was required.

Consent to Participate (Ethics)

Since the experiment was not conducted on humans, no consent to participate was required.

Consent to Publish (Ethics)

This study has not used any secondary data for publication, hence no consent to publish was required.

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Tables

Table 1. Estimate of genetic diversity in COI gene among *T. gigas* samples collected from Odisha.

Locality	Coordinates	Sample size	Numbers of Haplotypes	Haplotypes diversity (<i>H_d</i>)	Average number of nucleotide differences (<i>k</i>)	Nucleotide diversity (π)
Udaypur	21.588111 / 87.432111	7	2	0.571	0.571	0.00098
Chaumukh	21.566375 / 87.354022	1	-	—	—	—
Chandamani	21.549633 / 87.354022	3	2	0.667	0.667	0.00115
Kasafal	21.536691 / 87.173719	7	4	0.714	1.048	0.00180
Bahabalpur	21.515336 / 87.117036	12	6	0.682	1.212	0.00209
Balaramgadi	21.470663 / 87.071619	5	2	0.400	0.400	0.00069
Mahisali	21.409772 / 86.979433	12	6	0.818	2.848	0.00490
Khapara	21.375544 / 86.946691	7	5	0.905	2.095	0.00361
Khandia	21.338325 / 86.911961	76	15	0.700	1.146	0.00197
Kantiachira	21.315407 / 86.893259	12	6	0.864	1.636	0.00282
Chudamani	21.141586 / 86.786611	5	3	0.700	0.800	0.00138
Kanika Island	20.828744 / 86.988291	1	-	—	—	—
Dosinga	20.813338 / 86.964961	2	1	0.000	0.000	0.00000
Chandinipal	20.787877 / 86.959363	2	2	1.000	2.000	0.00344
		152	26	0.578	1.175	0.00265

Table 2. Variable nucleotide positions within 26 haplotypes of *T. gigas*.

Figures

Haplotype	Accession no.	1	2	4	6	7	7	1	1	1	1	2	2	2	2	3	3	3	4	4	4	4	5	5	5	5	5
		1	2	4	6	7	7	0	3	6	7	6	6	7	9	3	4	4	2	4	5	9	0	1	2	2	3
		8	6	3	0	6	8	3	8	2	3	2	5	2	5	4	0	6	9	2	3	9	8	9	2	5	6
H1	MZ262422	C	G	C	G	A	T	G	T	C	T	G	G	G	G	G	G	C	T	G	T	C	T	A	T	C	T
H2	MZ262423	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
H3	MZ262424	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
H4	MZ262425	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.
H5	MZ262426	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	A	.	.	.	.	.	.	.
H6	MZ262427	T	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H7	MZ262428	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.
H8	MZ262429	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.
H9	MZ262430	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H10	MZ262431	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
H11	MZ262432	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H12	MZ262433	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
H13	MZ262434	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.
H14	MZ262435	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H15	MZ262436	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
H16	MZ262437	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H17	MZ262438	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.
H18	MZ262439	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H19	MZ262440	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H20	MZ262441	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H21	MZ262442	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H22	MZ262443	.	.	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
H23	MZ262444	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	.	.	.	.
H24	MZ262445	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	.	.
H25	MZ262446	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
H26	MZ262447	.	.	.	A	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.

Figure 1

Location of 14 sampling sites along the coast of the Bay of Bengal in Balasore District, Odisha. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 2

Demographic parameters estimated from COI gene sequences for horseshoe crabs population in India. Mismatch analysis of 26 haplotypes of the horseshoe crab.

Figure 3

Phylogeographic tree for the COI fragment. The color represents the localities.

Figure 4

Chronogram based on the BEAST divergence time analysis showing the estimated evolutionary radiation of Asian horseshoe crabs. Blue bars indicate 95% confidence levels. The times scale below the chromatogram features all major geological periods in Mya.