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Original article

Study of genetic variation and phenetic relationships in some vulnerable taxa from Indian sundarbans following RAPD marker analysis

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ABSTRACT

The mangrove ecosystem plays an important role in maintaining coastal ecological balance and is also one of the most highly productive and dynamic ecosystems to mankind, providing food, livelihood and ecological security. The plant species of this ecosystem are constantly under environmental stress due to the highly saline conditions and extreme temperatures. Studying the genetic diversity of mangrove plants is important in taking effective measures to protect these species. The present study, being the first attempt in Indian Sundarbans, deals with RAPD (Random Amplified Polymorphic DNA) marker study of three IUCN declared 'Vulnerable' mangrove species viz. *Xylocarpus granatum*, *Xylocarpus mekongensis* and *Heritiera fomes* of Indian Sundarbans to assess its genetic diversity to trace their reduction in population size. These were collected from two distinct populations of Indian Sundarbans i.e. Sajnekhali Tiger Reserve and Bonnie Camp area. It was found that intra-specific polymorphism was highest (80%) in *Xylocarpus granatum* in and lowest (60 %) in *Xylocarpus mekongensis* while *Heritiera fomes* showed moderate (71.42 %) level of polymorphism. The decreasing

population size can be assessed through the low genetic variation of *Xylocarpus mekongensis* and *Heritiera fomes* rather than *Xylocarpus granatum*. It can further be said that comparatively high genetic variation of *Xylocarpus granatum*, though, does not exactly correspond to its population structure, it may be due to its isolated distribution, dissimilar edaphic factors and different anthropogenic activities within the mangroves of Indian Sundarbans. We can conclude that molecular marker study provides an effective tool to access the existing inter- and intra-specific genetic polymorphism within mangrove species of Indian Sundarbans and to explore their conservation strategy.

1. Introduction

Mangroves are perennial plants found in the intertidal zones of coastal wetlands in tropical regions; many species that are found exclusively there are called true mangroves and are capable of forming dense pure stands of trees. The mangrove ecosystem plays an important role in maintaining coastal ecological balance and is also one of the most highly productive and dynamic ecosystems to mankind, providing food, livelihood and ecological security. The plant species of this ecosystem are constantly under environmental stress due to the highly saline conditions and extreme temperatures and have become adapted to these frequent and fluctuating changes. Mangroves along the Indian coast have reached an alarming stage of depletion due to the absence of any national plan for conservation and sustainable use (Untawale, 1985). Detailed studies of these plants, including physiological, cytological, cytochemical and molecular aspects, are lacking in all the major areas. The substantial accumulation of wax, latex, phenolics and other secondary metabolites in the leaves of these plants makes it difficult to isolate nucleic acids for molecular work.

The mangrove genus *Xylocarpus*, belonging to the family Meliaceae, has three distinct species: *X. granatum* Koen., *X. moluccensis* Lamk. And *X. mekongensis* Pierre., that are distributed in tropical tidal forests of Old World, typical mangrove habitat or in sandy or coastal habitats spread from Africa to Australia, including India and Malayan Archipelago. In India, these three species were recorded from Andaman Islands and Orissa Coast whereas the two species, *X. granatum* Koen. and *X. mekongenesis* Pierre. were reported from Sundarbans, Tamil Nadu Coast and Andhra Pradesh. On the other hand, Heritiera fomes (Sterculiaceae) is a genus largely of tree mangroves associated with estuarine ecosystems and is distributed from eastern tropical Africa and India to the Pacific. This species has been reported from the Sundarbans, West Bengal (Banerjee, Sastry and Nayar, 1989), as well as the Bhitarkanika and Mahanadi delta of Orissa in India (Das, Basak and Das, 1994).

Due to overexploitation, habitat destruction and global warming mangroves are being destroyed at an alarming rate and has resulted in the loss of genetic diversity. To overcome these losses, conservation and sustainable management is, thus, a major priority. However, the genetic structure of plant species within the mangrove ecosystem of Sundarbans is poorly understood. Studying the genetic diversity of mangrove plants is important in taking effective measures to protect these species. Genetic studies are, therefore, aimed at providing the information needed for afforestation, domestication and for the conservation of genetic resources. Molecular markers can be used to assess polymorphism in this mangrove species to identify and detect distinct genotypes for long-term conservation. Development of molecular methods has opportunities to take mangrove research in new directions and to address unresolved issues in mangrove studies. Molecular markers like random amplified Polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) are extensively used to quantify the inter-specific, intra-specific and inter-generic variability in different plant groups including mangroves and crop varieties (Chalmers et al., 1994; Lin et al., 1996; Garcia-Mas et al., 2000; Sharma et al., 2000; Lakshmi et al., 2002; Mukharjee et al., 2003; Jena and Das, 2006; Kader et al., 2012, Begum et al., 2013; Hazarika et al., 2013). Das et al. (2001) reported molecular phylogeny of *Heritiera* Aiton (Sterculiaceae), a tree mangrove by the analysis of variations in RAPD markers and nuclear DNA content. In 2004, Jian et al. studied the variation in inter-simple sequence repeat (ISSR) in mangrove and non-mangrove populations of *Heritiera littoralis*. Jugale et al. (2009)

assessed genetic diversity in intra- and inter-population of *Xylocarpus granatum* using ISSR markers and found the low genetic variation in this species. Recently in 2013, Pawar et al. studied the genetic variation between *Xylocarpus* spp. revealed by RAPD markers.

The present study, being the first attempt in Indian Sundarbans, deals with threee mangrove species viz. *Xylocarpus granatum*, *Xylocarpus mekongensis* and *Heritiera fomes* of Indian Sundarbans to assess its genetic diversity. The main reason behind the selection of these three true mangrove species was its IUCN 'Vulnerable' status among Indian mangroves (Bhatt and Kathiresan, 2011). According to their report, distribution of *H. fomes*, *X. granatum* and *X. mekongensis* is rare (1-30%), common (31-60%) and frequent (61-80%) respectively among the mangrove population of Indian Sundarbans. Our study relates to the findings of the cause of its reduction in population size in Indian Sundarbans where these species were predominant taxa in early years. These three species were collected from two distant population of mangroves viz. Sajnekhali Tiger Reserve ($22^0$08'18.58"N, $88^0$53'12.71"E) and about 55.2 km distantly located Bonnie Camp area ($21^0$41'29.64"N, $88^0$34'05"E) of Indian Sundarbans. To study whether any inter-population variation exists causing reduction in population size of these species To study the variations at the molecular level within two different mangrove populations of these species, the RAPD technique was used. This marker study provides an effective tool to access the existing Inter- and Intra specific genetic variation within these species to design their conservation strategy.

2. Materials and methods

The materials were the fresh leaves of the plants that were taken for the molecular marker analysis. These three species were collected from two distant population of mangroves viz. Sajnekhali Tiger Reserve ($22^0$08'18.58"N, $88^0$53'12.71"E) and about 55.2 km distantly located Bonnie Camp area ($21^0$41'29.64"N, $88^0$34'05"E) of Indian Sundarbans (Figure 1).

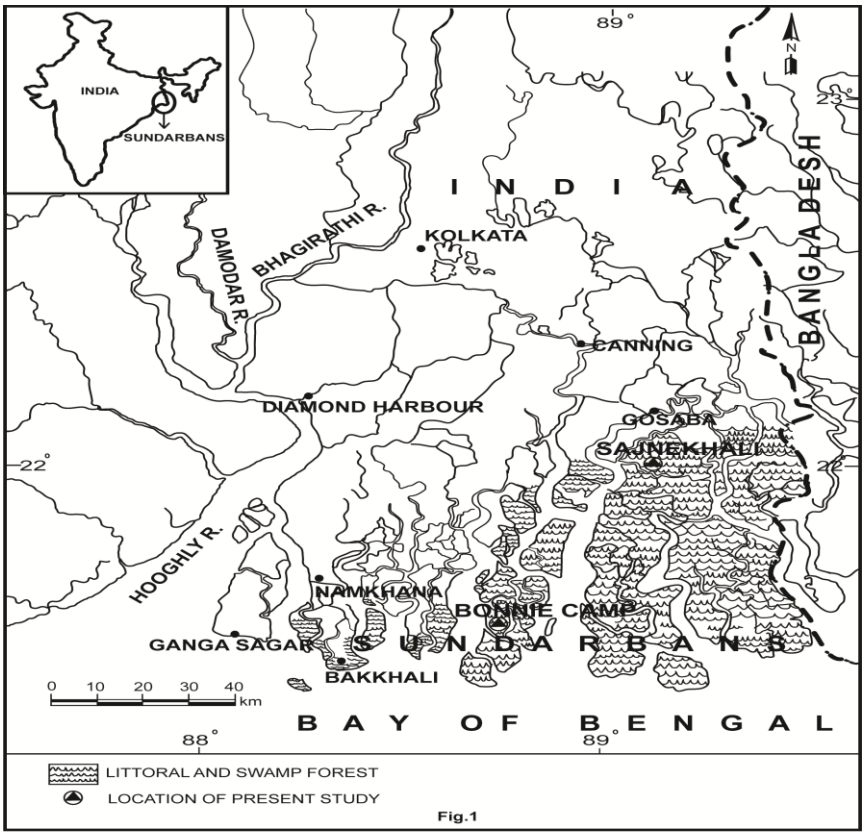

Fig.1. Map of Sundarbans, India showing study area.

The three species collected from Sajnekhali Tiger Reserve were designated as XG1, XM1 and H1 and that of from Bonnie Camp area were designated as XG2, XM2 and H2.

2.1. Extraction of genomic DNA

In all cases, DNA was extracted from fresh leaves. DNA was extracted either immediately after collection or were stored at -70°C until extraction. In both the cases, DNA was extracted following the CTAB method of Doyle and Doyle (1987) with minor modifications. 1 gm of frozen leaf sample was grinded with liquid Nitrogen and the leaf powder was then immediately transferred to a 50 ml tube containing 10ml 2% CTAB buffer, mixed properly and incubated for 1 hour at 65°C with occasional gentle swirling. After incubation, the mixture was centrifuged for 10 min at 4,000 rpm at room temperature and the supernatant was taken. It was treated with RNase for 30 min at 37°C. The equal volume of Phenol: Chloroform: Isoamyl Alcohol (25:24:1) mixture was added to the solution, mixed thoroughly, centrifuged at 4,000 rpm at room temperature and the supernatant was taken carefully. Double amount of isopropanol was added, incubated overnight at -20°C and centrifuged at 10,000 rpm (12,520 x g) at room temperature. The supernatant was discarded and the pellet was washed with 70% ethanol and again centrifuged at room temperature for 10 min at 10,000 rpm, dried using a vacuum desiccator. The pellet was re-suspended with 50 µl deionised doubled distilled water and allowed to dissolve and stored in -20°C for further use.

2.2. RAPD amplification

Primarily, one decamer primer 5'-MGGACTCGNTAACACGCA-3' was tested for RAPD amplification. Amplification reactions were performed based on the standard protocol of Williams et. al. (1990) with some modifications for optimized use with these mangrove species. Varied concentrations of Mg2+ (1.0, 1.5. 2.0, 2.5, 3.0 and 3.5 mM), template DNA (20, 30. 40, 50 and 60 ng per reaction mixture of 25 µl), Taq polymerase (0.5, 1.0, 1.5 and 2 Units per reaction mixture of 25 µl) as well as annealing temperature of primers and annealing time were tested. The optimum reaction mixture for a 25 µl contained 2.5 µl of 10X PCR buffer, 0.1 mM dNTP mix, 200 nM primer, 1U Taq Polymerase, 50 ng template DNA and 3mM MgCl2 in all the cases. The optimum amplification cycle was as follows: Initial denaturation at 94oC for 5 minutes, denaturation at 94oC for 1 minute, 45 cycles for Annealing at 36oC for 30 seconds, extension at 72oC for 1 minute and Final extension at 72oC for 5 minutes. Products were stored at 4oC until further analysis.

Amplification reactions were carried out in a Perkin Elmer Thermal Cycler 2400. The amplification products were size separated by AGE (Agarose Gel Electrophoresis) in 1.4% gel along with two molecular weight markers (λ DNA Hind III digest and ΦX 174 DNA Hae III digest mix and 100 bp DNA ladder) in 0.5X TBE (0.45 mM Tris-borate, 1 mM Na2EDTA). Then the gel was visualized under UV light following Ethidium Bromide staining. The gels were photographed with a Vilber-Laurmat gel documentation system. Molecular weights of amplified bands were calculated with Photo-Cap MW software package supplied with the gel documentation system.

2.3. Phenetic analysis

For phylogenetic analysis, each amplified band was treated as a unit character regardless of its intensity and scored in terms of a binary code, based on presence (1) and absence (0) of bands. Only clear and reproducible bands were considered for scoring. The three species of different locations were used in the present investigation. To analyze data obtained from the binary matrices, the NTSYS-pc version 2.1 statistical package (Rohlf, 2000) was used. One dataset was used for RAPD only. The statistical method took into account the presence or absence of each band as differential features. The binary qualitative data matrices were then used to construct similarity matrices. Among the various similarity matrices, primarily two different Jaccard (Jaccard, 1908) and Dice (Dice, 1945) similarity coefficients were chosen. The similarities were calculated with two coefficients are as follows:

Jaccard coefficient = a/2a+b+c

Dice coefficient = 2a/2a+b+c

Where a = number of bands present in both specimens;

b = number of bands present in the second but not in the first specimen;

c = number of bands present in the first but not in the second specimen;

d = number of bands absent in both specimens.

n = total number of scorable bands

The similarity matrices were then used to construct dendrograms following Unweighted Pair Group Method with Arithmetic Average (UPGMA) and Sequential Agglomerative Hierarchal Nested (SAHN) cluster analysis.

Bootstrapping was done using the software program 'Winboot' (Yap and Nelson, 1996) downloaded from the website www.irri.org/science/software/winboot.asp. To choose the best similarity index for this purpose, Cophenetic matrices were derived from the dendrograms using the COPH (cophenetic values) program, and the goodness-of-fit of the clustering to the two data matrices were calculated by comparing the original similarity matrices with the cophenetic value matrices using the Mantel matrix correspondence test (Mantel, 1967) using the MXCOMP program. Principal co-ordinate analysis (PCOORDA) was performed based on the similarity coefficients using DCENTER module to transform the symmetric similarity matrix to scalar product form and then EIGEN module was used to extract eigenvectors resulting into a three dimensional plot showing the taxa in a three dimensional space. Additionally, RAPD based trees were summarized by Strict consensus method (Sokal and Rohlf, 1981) using the CONSEN module of NTSYS-pc. Lastly, a combined dataset was prepared using RAPD data and used to calculate the combined similarity matrix which was ultimately used to construct phylogenetic tree and Principal co-ordinate analysis. The combined phylogenetic tree was compared with RAPD based trees using the Mantel matrix correspondence test (Mantel, 1967).

3. Results

3.1. Analysis of RAPD profiles

The selected primer produced highly reproducible and polymorphic banding patterns across all the investigated taxa. The taxa varied considerably in band number. The brief descriptions of the banding patterns are given below.

The primer produced total 17 bands and 6 banding patterns (Figure 2). The highest and the lowest number of bands were produced by H1 (6 bands) and XG2 (1 band) respectively. All the bands were polymorphic. Thus, every species was represented by their characteristic banding patterns. There were several species specific bands. For example, among *Heritiera* species, H1 produced 4 species specific bands while H2 produced only one species specific band. XG1 produced 2 species specific bands. XM2 produced 2 species specific bands.

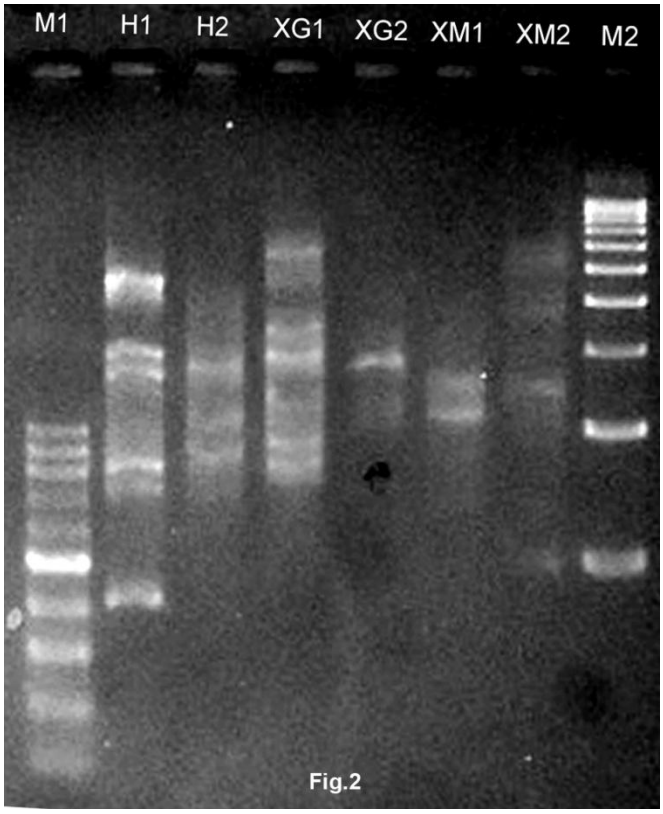

Fig.2. RAPD Agarose Gel Photograph.

3.2. Genetic variation and levels of polymorphism as revealed with RAPD profiles

The RAPD primer produced total 17 bands in all the investigated species studied. All of the loci were polymorphic at the genus level. However, *intra-specific polymorphism was highest (80%) in Xylocarpus granatum in and lowest (60 %) in Xylocarpus mekongensis* while *Heritiera* showed moderate (71.42 %) level of polymorphism. Additionally, there was one band that has been shared between *Heritiera* and *Xylocarpus mekongensis*.

3.3. Phenetic relationships

The phenetic analysis included 6 for giving much more complete information. Primarily, two different coefficients viz. Jaccard, and Dice coefficients were tested to prepare the similarity matrix between all 31 taxa. However, goodness-of-fit test of the matrices with their corresponding dendrograms revealed that Jaccard's coefficient was the best fit for this analysis. The goodness-of-fit of both Jaccard and Dice coefficients were 0.89791. The correlation of Jaccard and Dice coefficients was 1.00 (Figure 3). Similarity matrices of both Jaccard and Dice were the same. Similarity matrix based on Jaccard's co-efficient is presented in Table 1.

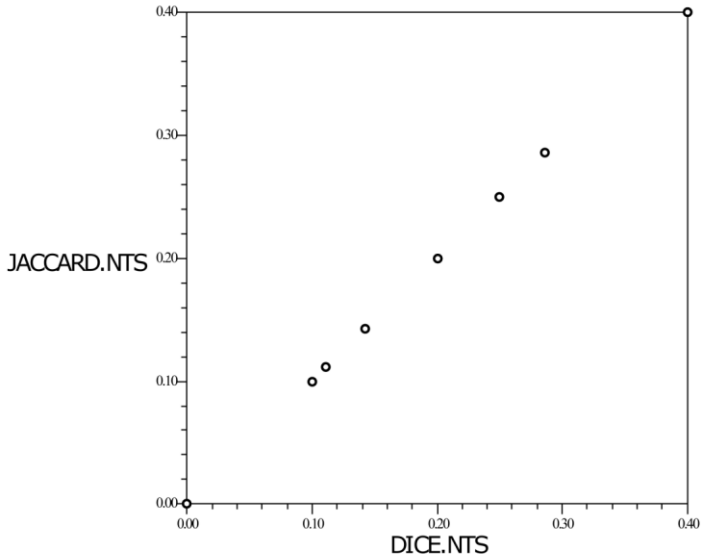

Fig.3

Fig.3. Correlation of Jaccard and Dice Co-efficients.

Table 1
Similarity matrix based on Jaccard's co-efficient.

	H1	H2	XG1	XG2	XM1	XM2
H1	1.0000					
H2	0.2857	1.0000				
XG1	0.1000	0.1429	1.0000			
XG2	0.0000	0.0000	0.2000	1.0000		
XM1	0.1429	0.2500	0.0000	0.0000	1.0000	
XM2	0.1000	0.1429	0.1111	0.0000	0.4000	1.0000

Inter-specific variation was high among all species as revealed by the very low similarity values. Among the three species, intra-specific genetic diversity was highest in XG1 and XG2 (J = 0.2). The lowest diversity was found within XM (J = 0.4). Heriteria showed moderate genetic diversity (J = 0.2857).

The UPGMA dendrogram showed the phenetic relationship among the taxa. All three species were clearly separated in this dendrogram (Figure 4). However, the taxa of *X. mekongensis* grouped together at J = 0.40, taxa of

X. granatum grouped together at approximately J = 0.20 and taxa of *Heritiera* grouped at J = 0.27. This reflects the Jaccard similarity matrix discussed above. The three dimensional plot of Principal Coordinate Analysis (Figure 5) showed the arrangement of the six taxa according to their similarity. The first three eigenvalues described 65.6404% variation among the taxa. The three dimensional plot showed that different taxa within the different species arranged themselves together. Here also, XM1 and XM2 showed more closeness than the other species.

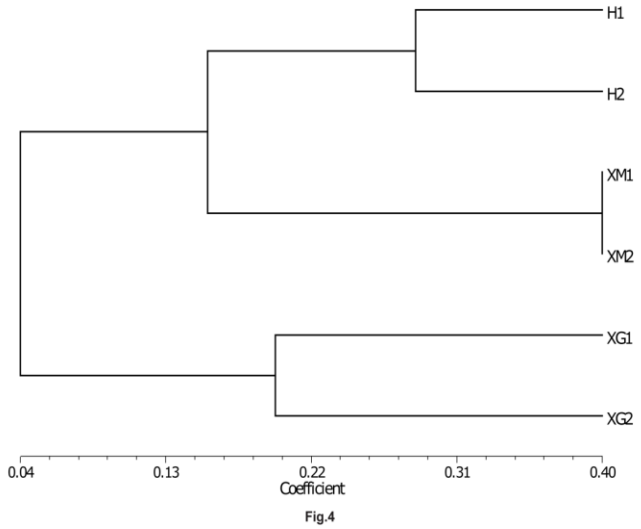

Fig.4. UPGMA dendrogram.

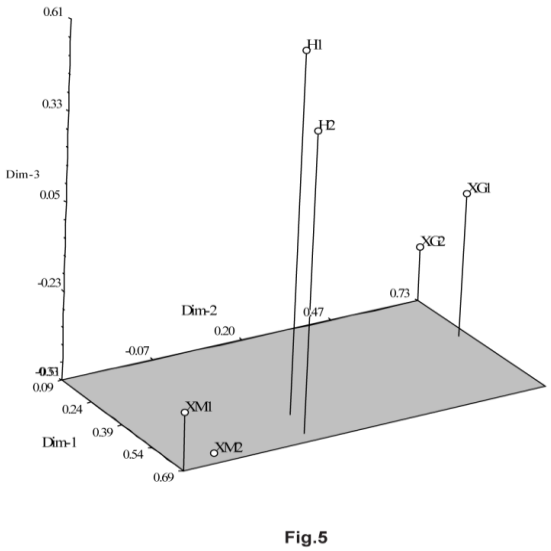

Fig.5. 3-D plot of Principal Coordinate Analysis.

4. Discussion

This study showed that RAPD along with proper statistical tools can be successfully applied for the study of genetic diversity of mangrove species. This study also showed that intra-specific polymorphism between two

populations of *X. mekongensis* was the lowest among the three species. It revealed that XM1 and XM2 were more genetically close to each other, which ensured its low genetic variation or diversity. But being in the same genus, *X. granatum* showed higher genetic diversity having the highest polymorphism within XG1 and XG2. On the other hand, *H. fomes* showed moderate level of polymorphism within H1 and H2 causing moderate level of genetic diversity. So, in terms of population, it can be said that though inter-population variation was lower in *X. mekongensis* and *H. fomes*, it existed in higher amount in *X. granatum*.

The low genetic variation of *X. mekongensis* clearly showed that low genetic diversity may be the cause of reduction in its population size. The moderate percentage of polymorphism within two populations of *H. fomes* also indicated its downwards genetic diversity in Indian Sundarbans. The data regarding *X. granatum* nearly coincided with the result of polymorphism reported by Pawar et al., 2013 that rules with the exception cited by Jugale et al., 2009.

The genus *Xylocarpus* and *Heritiera*, once predominant taxa, have lost their abundance in Indian Sundarbans now. The dominance of *Xylocarpus* was recorded by its palynological analysis during ca. 8420-7560 cal yr. BP. (Hait and Behling, 2009) and the abundance of *Heritiera* as dominant taxon was revealed by its pollen record and in situ tree trunk during ca. 9880-5000 cal yr. BP. (Gupta, 1981; Sen and Banerjee, (1990, 1995); Hait and Behling, 2009).

It is evident that the decreasing population size of *X. mekongensis* and *H. fomes* can be assessed through its low genetic variation. But the IUCN status of *X. granatum* does not correspond to its polymorphism in Indian Sundarbans. Loss of individuals or population at certain locations may not cause immediate loss in genetic diversity, it may be a more long term effect. Otherwise, genetic variation may not be the prime cause for the low population of any individual species within mangrove ecosystem. The reduction in population size of this taxon may be due to its isolated distribution, dissimilar edaphic factors, anthropogenic activities like cutting of woods for fuel and human settlement within the mangroves of Indian Sundarbans. The present study was done using only one DNA primer during molecular marker analysis, it will be more effective after going through several primers analysis. On the basis of our results, we can conclude that molecular marker study provides an effective tool to access the existing inter- and intra-specific genetic polymorphism within mangrove species of Indian Sundarbans and to explore their conservation strategy. Along with other parameters, the total work is in progress.

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