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Genetic variation between *Xylocarpus* spp. (Meliaceae) as revealed by Random Amplified Polymorphic DNA (RAPD) markers

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Abstract

The genetic diversity of *Xylocarpus* a tree mangrove species was assessed. Three species viz., *X. granatum, X. moluccensis* and *X. mekongensis* were collected from different localities of the states of Tamil Nadu and Andhra Pradesh in India. These three species showed high degrees of phenotypic variation in the vegetative parts such as leaf, bark and root. To assess the variations at the molecular level within the three species of *Xylocarpus* a RAPD technique was used. A total of 283 DNA fragments were generated by 25 random primers, with an average of 11.32 easily detectable fragments per primer. A higher similarity was observed between *X. mekongensis* and *X. moluccensis*, whereas *X. granatum* and *X. mekongensis* showed lower similarity. *X. granatum* and *X. mekongensis* had a variation of more than 79 %. On the other hand, *X. mekongensis* and *X. moluccensis* seem to be closely related with 35% variation. These variations may be due to its genotypic variation, isolated distribution and adaptation to dissimilar edaphic and environmental factors. All the three species of *Xylocarpus* are related at various degrees. These results will be helpful in future to assess the existing interspecific genetic polymorphism in *Xylocarpus* species and to design strategy for their conservation.

Key words: Genetic diversity, *Xylocarpus*, RAPD

Introduction

Mangroves are unique plant communities inhabiting the estuarine and inter-tidal regions of both tropical and subtropical coasts. The total number of exclusive or true mangrove species in the world is 68 and they belong to 27 genera (Duke, 1992). The genus, *Xylocarpus*, belonging to the family Meliaceae has three distinct species, *viz*., *X. granatum* Koen., *X. moluccensis* Lamk. and *X. mekongensis* Pierre. These species are distributed in tropical tidal forests of old world, typical mangrove habitats or in sandy or in coastal habitats spread from Africa to Australia including India and Malayan Archipelago (Tomlinson, 1986). In India, these species were recorded from Andaman Islands, coastal line of Orissa, Andhra Pradesh and Pichavaram mangrove forest of Tamil Nadu in the East coast of India. *X. granatum* is distributed in West coast mangroves e.g. Maharashtra of India. *X. granatum* and *X. mekongensis* are moderately sized trees with a well-developed woody trunk, whereas *X. moluccensis* is a medium-sized crooked, much-branched evergreen tree up to 10 m tall. They are found on the fringes of backwater creeks. Usually this taxon is associated with *Avicennia, Excoecaria, Acanthus, Rhizophora* and *Bruguiera* (Raju, 2003). The bark of *X. mekongensis* is rich in tannin and is used for tanning heavy hides, toughening fishing-nets and dying cloth. The wood is of good quality and used for boat building, nails, house-posts, small objects, furniture and firewood. The seeds have medicinal properties. Molecular markers like random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism are extensively used to quantify the inter-specific, intra- specific and inter-generic variability in different plant groups and crop varieties (Chalmers et al., 1994; Lin et al., 1996; Garcia Mas et al., 2000; Sharma et al., 2000; Mukharjee et al., 2003; Begum et al., 2013). Parani et al. (1997) identified the parentage of *Rhizophora* hybrid, by establishing its maternal status using molecular markers such as RAPD and RFLP. Jian et al. (2004) analyzed the variation in inter simple sequence repeats (ISSR) in mangrove and non-mangrove populations of *Heritiera littoralis.* In 2004, Mukherjee and his

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associates studied the genomic relationship among nine mangrove and two non-mangrove species of India belonging to the family Rhizophoraceae and proved that mangroves and non-mangroves are related distantly. Su et al. (2006) studied genetic variation in the mangrove, *Lumnitzera racemosa*.. Triest (2008) reviewed the uses of dominant markers such as RAPD and AFLP for identifying the mangroves and studying their relationship. Jugale et al. (2009) assessed genetic diversity in intra and inter-populations of *X. granatum* using ISSR markers and showed that variation exists at the phenotypic level but the genetic variability among the populations of *X. granatum* is very low. The molecular markers are phenotypically neutral having no epistatic and developmental effects. Moreover, they can detect the variation in both coding and non-coding regions of the genome. Among the several types of markers RAPD markers have been successfully proven to distinguish the variations. There are no attempts made to study the genetic diversity of three mangrove species of the genus, *Xylocarpus* in peninsular India.

In the present study the genus *Xylocarpus,* a mangrove species has been assessed for genetic diversity. Three species viz., *X. granatum*, *X. moluccensis* and *X. mekongensis* were collected from different localities Tamil Nadu and Andhra Pradesh in India. These three species showed higher degrees of phenotypic variation in the vegetative parts such as leaf, bark and root. To assess the variations at the molecular level within three species of *Xylocarpus*, the RAPD technique was used. RAPD markers may help to find out the difference at the molecular level between these species, and which in turn will prove useful to explore the adaptation of these three species in different habitats.

Material and methods

Plant material and DNA extraction

The seeds of *X. granatum* and *X. moluccensis* were collected from Kothapalem (11º 51′ N and 80º 54′ E) of Andhara Pradesh and *X.mekongensis* from Pichavaram (11º 27′ N and 79º 47′ E) of Tamil Nadu. The physical characteristics of sampled location given below in Table 1. The seeds were collected during the monsoon season in the month of November- January (2010-2011). The seedlings of the 3 species (*X. granatum, X. mekongensis* and *X. moluccensis)* were raised in the Botanical garden, Department of Botany, Annamalai University. The morphological features of these three species were recorded (Table 2 and Figure 1) Genomic DNA was isolated from freshly collected leaves using the CTAB (cetyl trimethyl ammonium bromide) method (Saghai-Maroof et al., 1984) with some modifications. 0.5 g of young leaf tissue was ground in liquid Nitrogen to fine powder using sterile pestle and mortar and suspended in 750 µl of preheated 2% CTAB buffer (2% CTAB; 0.1 M Tris pH 8.0; 20 mM EDTA; 1.4 M NaCl; 2% Polyvinylpyrrolidine-40 and 1% β-mercapto ethanol). The suspension was incubated at 65°C for one hour with occasional inversion. An equal volume of chloroform: isoamyl alcohol (24:1) mixture was added to the suspension and centrifuged at 10,000 g for 15 minutes. The aqueous phase was transferred to a new microfuge tube and extracted with 0.2 volumes of 5% CTAB buffer and equal volume of chloroform: isoamyl alcohol (24:1) mixture at 10,000 g for 15 minutes. The Chloroform: IAA extraction step was repeated twice. The aqueous phase was transferred to a fresh tube containing 0.6 volumes of ice-cold isopropanol and incubated overnight at -20°C and subsequently, centrifuged at 10,000 rpm for 15 minutes, to recover the nucleic acids. The pellet was washed with 70% ethanol and air dried and dissolved in TE buffer (10 mM Tris; 1 mM EDTA, pH 8.0). The extracted DNA was quantified by using Nanophotometer (IMPLEN, GmbH, Munich, Germany) and diluted to 15 ng/ µl for PCR amplifications.

Evaluation of genetic diversity

The 25 RAPD 10-mers used (Table 3) were selected from among 134 RAPD primers (obtained from Operon Technologies USA) in a preliminary test for oligos that amplified numerous discrete fragments. RAPD profile of five primers across the three species is depicted in Figure 2. Every 15 µl reaction volume consisted of 15 ng of genomic DNA, 1.5 µl of 2mM each dNTPs, 1.5 µl of 10x Taq DNA Polymerase assay buffer, 1.8 µl of 15mM $MgCl_2$, 5 µM RAPD primers and 1 U Taq DNA polymerase (Fermentas). PCRs were performed using a in an Eppendorf Thermocycler and involved an initial denaturation step (94°C, 3 min), 40 amplification cycles (each 94°C, 30 s; 37°C, 30 s and 72°C, 60 s) and a final extension step (72°C, 15 min).

Table 1. Physical characteristics of sampled locations.

Location	Physical Characteristics		
	Soil pH	Salinity (ppt)	Soil type
Kothapalem (Andhra Pradesh)	7.5 – 8.3	6 – 14	Saline swampy
Pichavaram (Tamil Nadu)	7.8- 8.4	8 – 24	Fine and coarse silt

Table 2. General morphological characters to identify the 3 species of *Xylocarpus.*

Morphological characters	*X. granatum*	*X. mekongensis*	*X. moluccensis*
Leaves	Paripinnate leaves, light green in colour with a rounded apex, Elliptic /obovate leaflets	Paripinnate leaves having 1, 2 or 3 pairs of leaflets with pointed apex	Leaves paripinnate, long, glabrous; leaflets upto 12 × 5 cm, elliptic, oblong-elliptic or obovate-elliptic, apex usually rounded.
Bark	Trunk surface is pale, smooth with its thin bark peelings in flakes or patches	Trunk surface is dark brown, fissured with the bark peelings in long thick narrow strips	Bark smooth and yellowish or brown and green with flaking
Root	Horizontal cable roots develops into ribbon like plank roots	Vertical conical laterally compressed knee roots or pneumatophores present	Surface roots laterally compressed and forming a spreading network of ribbon like pneumatophores.
Flowers	Inflorescence is regular with 3 flowered cymes, Flowers small and white in colour	Inflorescence is lax upto 10 cm long or more, flowers creamy white in colour	Flowers whitish or pale pink, in lax racemes of 2-3 lowered cymes.
Fruit	Large globose upto 20-30 cm across	Subglobose 8-12 cm across	Large 10-15 cm across

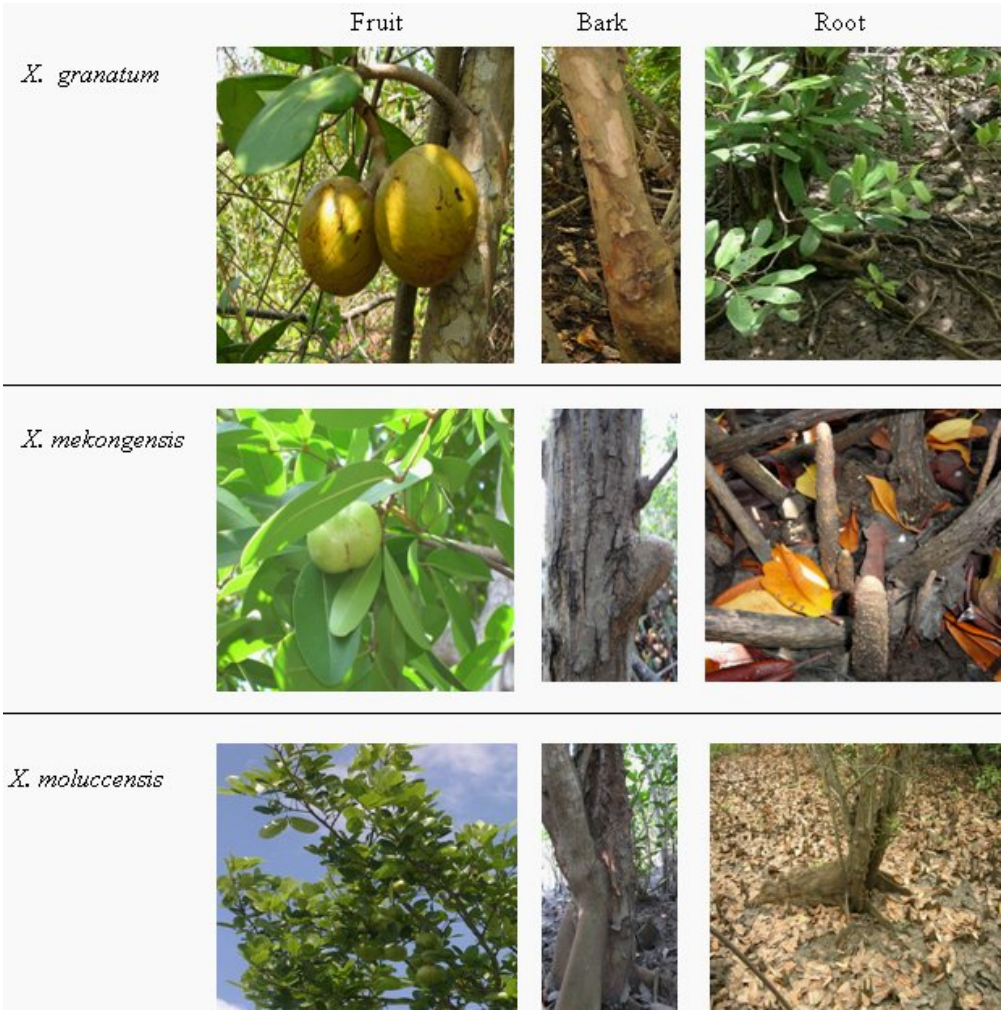

Figure 1. Morphological variations among the three species of *Xylocarpus.*

Table 3. Details of the random primers used for the genetic relationship studies across three species of genus *Xylocarpus.*

Marker	Sequence (5' – 3')	Total no. of loci amplified	Range of amplicons (bp)
OPK04	CCGCCCAAAC	15	240 – 1480
OPK08	GAACACTGGG	7	500 - 3000
OPL01	GGCATGACCT	16	250 - 3500
OPL05	ACGCAGGCAC	12	550 - 2000
OPL07	AGGCGGGAAC	8	600 - 1900
OPL18	ACCACCCACC	8	680 - 2500
OPL19	GAGTGGTGAC	14	600 - 6000
OPM10	TCTGGCGCAC	14	500 - 3700
OPM11	GTCCACTGTG	3	750 – 2250
OPM20	AGGTCTTGGG	11	300 - 2500
OPQ20	TCGCCCAGTC	12	350 - 3000
OPT02	GGAGAGACTC	15	400 - 3000
OPW05	GGCGGATAAG	18	250 - 3000
OPW08	GACTGCCTCT	11	400 - 6000
OPX01	CTGGGCACGA	14	400 – 2500
OPX02	TTCCGCCACC	12	400 - 2500
OPX03	TGGCGCAGTG	16	350 - 3000
OPX15	CAGACAAGCC	12	300 - 2250
OPY01	GTGGCATCTC	8	625 - 3500
OPY02	CATCGCCGCA	7	470 - 2000
OPY03	ACAGCCTGCT	10	280 – 1520
OPY05	GGCTGCGACA	7	580 - 1500
OPY06	AAGGCTCACC	7	550 - 1600
OPZ14	TCGGAGGTTC	11	525 - 1800
OPZ15	CAGGGCTTTC	15	400 - 2200
Total		283	-

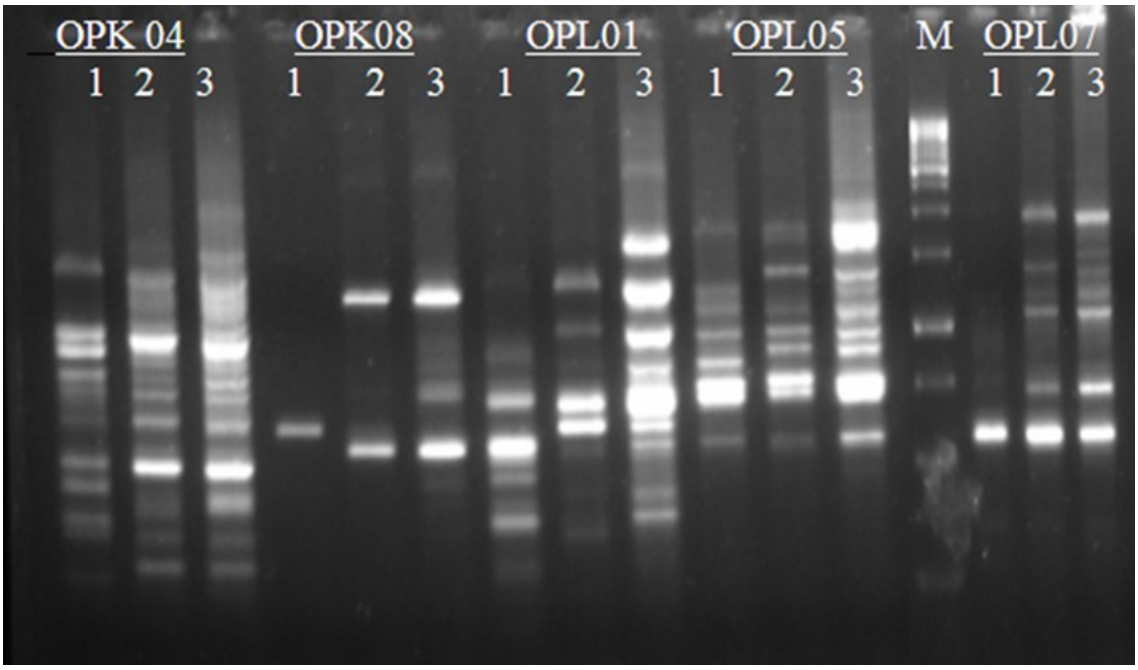

Figure 2. RAPD profile of three species of *Xylocarpus.*

Table 4. Details of the RAPD analysis with the three *Xylocarpus* species.

Marker	Total No. of loci obtained			No. of polymorphic loci			% polymorphism		
	Xg	Xme	Xmol	Xg	Xme	Xmol	Xg and Xme	Xme and Xmol	Xg and Xmol
OPK04	8	8	9	6	6	7	85.71	45.45	69.23
OPK08	1	3	6	1	3	6	100.00	50.00	100.00
OPL01	6	6	11	4	4	9	81.81	69.23	78.57
OPL05	7	7	9	3	3	5	60.00	45.45	33.33
OPL07	2	6	7	0	4	5	66.66	37.50	71.42
OPL18	3	4	6	2	3	5	83.33	57.14	71.42
OPL19	8	7	7	6	5	5	84.61	25.00	84.61
OPM10	8	6	6	8	6	6	100.00	0	100.00
OPM11	1	3	3	0	2	2	66.66	0	66.66
OPM20	5	6	7	4	5	6	90.00	14.28	90.90
OPQ20	11	2	3	10	1	2	91.66	33.33	83.33
OPT02	10	3	12	8	1	10	81.81	75.00	53.33
OPW05	11	6	11	10	5	10	93.75	45.45	77.77
OPW08	9	1	4	8	0	3	88.88	75.00	81.81
OPX01	9	10	11	3	4	5	61.53	9.09	57.14
OPX02	1	11	2	0	10	1	90.90	91.66	50.00
OPX03	3	14	6	2	13	5	93.75	66.66	71.42
OPX15	6	6	6	6	6	6	100.00	0	100.00
OPY01	5	6	6	2	3	3	62.50	0	37.50
OPY02	2	6	4	1	5	3	85.71	33.33	80.00
OPY03	4	9	6	2	7	4	70.00	33.33	75.00
OPY05	5	5	5	2	2	2	71.42	0	57.14
OPY06	6	5	5	2	1	1	42.85	0	42.85
OPZ14	7	8	4	7	8	4	63.63	50.00	100.00
OPZ15	7	11	9	5	9	7	73.33	18.18	85.71
Total	145	159	165	102	116	122	-	-	-
Average	5.8	6.36	6.6	4.08	4.64	4.88	79.62	35	72.76

Xg = *X. granatum* Xme = *X. mekongensis* Xmol = *X. Moluccensis*

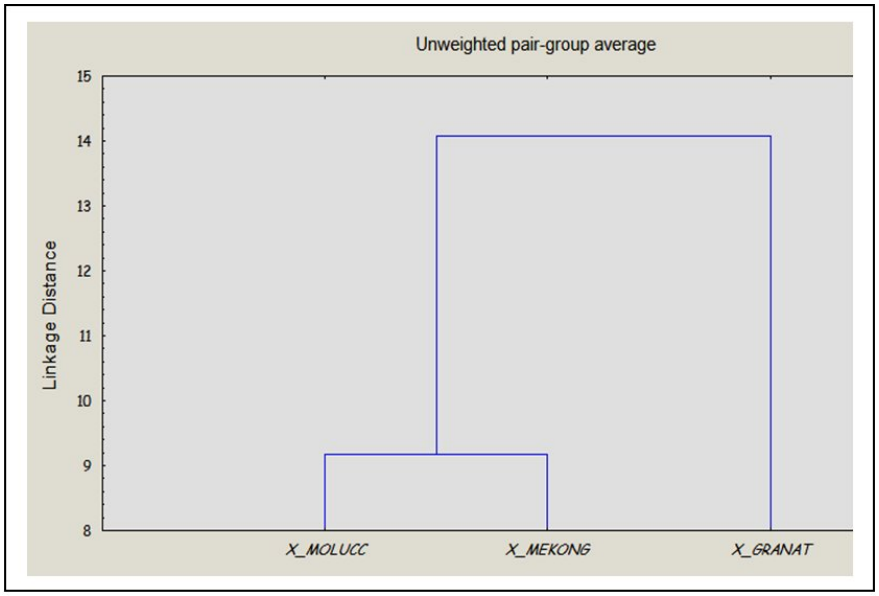

Figure 3. UPGMA dendrogram showing the relationship among three species of *Xylocarpus*.

Data analysis

The amplified products were separated on 1.5% (w/v) agarose gels by electrophoresis in 1X TBE buffer and visualized under UV light after ethidium bromide staining. To confirm the reproducible amplification of scored fragments, all amplifications were repeated twice. All the visible RAPD fragments were counted for each primer (Table 4) and robust polymorphic bands were scored as present (1) or absent (0) for each sample. For each primer, the number of polymorphic bands was calculated. The statistical analysis was carried out using the STATISTICA program (version 4.5 Stat soft Inc, USA). A phylogenetic dendrogram (Fig 3) was obtained by cluster analysis following the Unweighted Pair Group with Arithmetic Mean (UPGMA) method.

Results and Discussion

A total of 25 random primers were used for RAPD analysis with the 3 species of *Xylocarpus.* Majority of these primers showed polymorphic bands across the three species. PCR amplification using the genomic DNA of three species of *Xylocarpus* with 25 RAPD primers produced a total of 283 bands, of which, 242 bands were polymorphic. The size of the amplicons ranged from 240 to 6000 basepairs. Mangroves are constantly subjected to physiological stress caused by fluctuating growing conditions (Chapekar, 1994). Despite such extremes, they have successfully colonized suitable areas by developing morphological, physiological and reproductive adaptations (Clough et al., 1982; Clough et al., 1994; Saenger, 1982). Therefore depending on the genetic architecture of these species and their edaphic preferences and adaptations, different species are likely to display varying degree of polymorphism. Present observations on *Xylocarpus granatum* do support this presumption. In the three species, a maximum of 18 loci was obtained with the primer-OPW5, while the primer OPM11 resulted in a minimum of 3 loci. The total RAPD loci between the *Xylocarpus* species for individual primers differed according to the genomic characteristics of the species and the total number of amplicons resolved was 165 in *X. moluccensis*, followed by 159 in *X. mekongensis* and 145 in *X. granatum*. Ge and Sun (1999) studied genetic variation within and among populations of *Aegiceras corniculatum.* They recorded very low variation and low gene differentiation among populations. Huang et al. (2008) assessed interspecific and inter-population variation in three species of *Ceriops* and recorded low-genetic diversity at population level.

The average number of bands per primer varied from 5.8 to 6.6 in the species of *Xylocarpus*. The number of polymorphic loci ranged from a minimum of 102 in *X. granatum* to a maximum of 122 in *X. moluccensis* with an average ranging from 4.08 to 4.88 with the 25 random primers. It has been reported by Mukharjee et al. (2005) that nine members belonging to Rhizophoraceae formed a single cluster with RAPD analysis. The maximum average percent polymorphism of 79.62% was observed between the species of *X. granatum* and *X. mekongensis*, whereas the minimum DNA polymorphism of 35% existed between *X. mekongensis* and *X. moluccensis* and it was 72.76% with *X. granatum* and *X. moluccensis.* The cluster analysis of the RAPD profile with 25 primers following the method of Unweighted Pair Group with Arithmetic Mean (UPGMA) revealed that the 3 species of *Xylocarpus* clustered into two distinct branches of a single tree. *X. mekongensis* and *X. moluccensis* clustered together with a linkage distance of 9.2, whereas *X. granatum* is 4.8 linkage distance away from the above two species. *Xylocarpus mekongensis* and *Xylocarpus moluccensis* formed a single cluster while *X. granatum* formed different cluster. All the three species of *Xylocarpus* are related at various degrees, while *X. granatum* is distantly related with *X. mekongensis* and *X. moluccensis*. The *X. mekongensis* and *X. moluccensips* seem to be closely related and have 35% variation. This result coincides with the morphological and floristic variation exists among the species. *Xylocarpus granatum* is a critically endangered species of mangrove along the Indian coastline disappearing from many locations and represented by few individuals (Jugale et al., 2009). Loss of individuals or populations at certain locations may not cause immediate loss in genetic diversity, but more damage may occur in terms of long term genetic consequences due to the reduced numbers of populations and smaller population sizes. These variations may be due to isolated distribution and adaptation to dissimilar edaphic and environmental variation exists within the mangroves of Peninsular India.

In conclusion, our results demonstrate that molecular markers provide an effective tool to access the existing interspecific genetic polymorphism in mangrove species and to design their conservation strategy. The Mangrove genus *Xylocarpus* having high genetic variability may be

due to varied edaphic and climatic conditions of various mangroves localities.

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