Chromosome Stability and Inter-population Genetic Variability in a Tree Mangrove *Xylocarpus granatum* Koen. (Meliaceae) as Revealed by RAPD Markers

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Received September 12, 2007; accepted March 31, 2008

Summary Detailed chromosome analysis of 8 populations of *Xylocarpus granatum* showed a constant somatic chromosome numbers of $2n=48$ with a very symmetric karyotype. Structural details recorded no such major variation in the chromosome morphology. Random amplified polymorphic (RAPD) markers were used for determining the genetic diversity among 8 populations of this minor tree mangrove species. Individuals collected from several sites in north-east coast of India were analyzed for their genetic relationship. A total 444 amplicons were generated among the ecotypes out of which ~67% bands were found polymorphic. Inter population polymorphism among 8 populations of *X. granatum* varied between 36.25 to 72.5%. Clustering based on similarity index was done following Unweighted Pair Group with Arithmetic mean (UPGMA) method and intra-genetic relationships were analysed. The obtained dendrogram of the RAPD data showed prominent patterns of interpopulation relationships that are discussed in the light of the respective habitats of the ecotypes distributed in different mangrove pockets of the north-east Indian sea coast. It was evident from RAPD data that high degree of divergence exists among 8 studied populations, which suggests their adaptation in various microclimatic condition is suitable for the specific habitat of a particular ecotype.

Key words Karyotype, Mangrove, RAPD markers, Somatic chromosome, *Xylocarpous*.

The widespread loss of the world's biological diversity is one of the most serious global crises today. Conservation of the endangered plant species is the most effective means of preventing the crisis. When an endangered species is vegetatively propagated which is located in a state of collection of few genotypes, collection of individuals for conservation purpose becomes critical. Because of the long-term life cycle, tree genetics tends to receive less attention than annual crops. Among tree species, mangrove plants have not so far been subjected to detailed genetic analysis (Balakrishna 1995). Mangrove forests form the interface between marine and terrestrial environments and stretch from the inter-tidal zone up to the high-tide mark. These forests are comprised of twelve genera comprising about 60 species of salt-tolerant trees. Out of several cosmopolitan species of mangrove, *Xylocarpus granatum* of the family Meliaceae is an important species because it withstand varying soil conditions, high level of salinity, and lack of freshwater inflow for considerable periods of time and pollution of varying forms (Scholander *et al.* 1955). Because of their intertidal location, *Xylocarpus granatum* have developed a number of morphological and physiological adaptations to cope with the salt water from the ocean and live within specific zones in their ecosystem. Genetic characterization of mangroves is unfortunately limited owing to lack of concerted studies on identifying the genetic variability within species. This species produces gigantic seeds only of 2

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Table 1. Genotypes of *X. granatum* from different populations with their physical characteristics

Source of collection	Population	Latitude/longitude	Physical characteristics of different populations			Number of individual plants analysed for RAPD analysis
			Soil pH	Salinity (ppt)	Soil type	
Baighar, Orissa	Pop-I	20°17'12"N/86°18'24"E	6.4-6.9	6-22	Sandy and clayey	5 individual plants
Kajalapatia, Orissa	Pop-II	20°17'12"N/86°42'43"E	6.8-7.3	7-20	Dark-clayey soil	3 individual plants
Bhitargarh, Orissa	Pop-III	20°17'54"N/86°42'12"E	6.3-7.0	7-20	Fine silt/clay	4 individual plants
Bhitarkanika, Orissa	Pop-IV	20°40'35"N/86°52'24"E	6.7-7.6	7-25	Silt soil	3 individual plants
Kalibhanjadia, Orissa	Pop-V	20°47'46"N/86°54'17"E	6.1-7.2	5-18	Sandy Clayey Soil	2 individual plants
Sunderban, W. B.	Pop-VI	21°56'01"N/88°51'01"E	6.7-7.8	6-21	Clayey, compact	2 individual plants
Machilapatnam, A. P.	Pop-VII	16°10'35"N/81°08'24"E	6.8-7.4	8-19	Clayey, compact	4 individual plants
Pondicherry	Pop-VIII	12°00'04"N/80°51'01"E	6.7-8.3	6-19	Fine silt/clay	2 individual plants

to 3 with in a fruit due to cross pollination and fruits are not produced so profuse; regeneration is limited with a hurdle of various biotic and abiotic factors. Since all the regenerations are of seed origin it is therefore essential to find out its genetic variability for better understanding of ecological management of this threatens species in various climatic conditions. There is no report on the chromosome number as well as a DNA marker study in analyzing genetic diversity of *Xylocarpous* except for very few species of mangroves like *Acanthus*, (Lakshmi *et al.* 1997, Jena *et al.* 2002), *Avicennia* (Parani *et al.* 1997), and *Heritiera* (Das *et al.* 2001). For evaluation of genetic diversity in species where cloned markers are not available, RAPDs are better choice of markers. Hence, RAPD analysis was undertaken in *Xylocarpus granatum* that showed clear inter-population variability from the collections made in Orissa and West Bengal of India being a home of complex and varied vegetation structure for conducive growth of diverse floral elements. This present study describes the result of a molecular-marker-based analysis (RAPD) of a minor mangrove, *Xylocarpus granatum* along with the cytological analysis, which is present as distinct 8 populations along the coastline of North East India; edged by the distance which is a common measure used to indicate the intensity of habitat fragmentation.

Materials and methods

Plant materials

Xylocarpus granatum Koen populations were collected from eight different sites from North-East Indian sea coast; the name of populations, their source of collection, salinity range, soil type and location details have been depicted in Table 1. An equal quantities of young leaf sample from single plant of each populations were collected separately which is demarked as Accessions and stored in a -85°C freezer for DNA extraction.

Cytological studies

Young and healthy root-tips were pretreated in saturated paradichloro-benzene and aesculine mixture for 4 h at 18°C followed by overnight fixation in propionic acid: ethanol (1:3). Chromosome staining was done in 2% propionic orcein after cold hydrolysis in 5 N HCl for 7 min. Root-tips were squashed in 45% propionic acid. Well-scattered 10 metaphase plates were selected for karyotype analysis of each species. The total chromosome length was ascertained by adding the length of all

Table 2. Detailed karyotype of *X. granatum* Pop-VI collected from Sundarbans showing somatic chromosome characteristics

Types of chromosome	Number of chromosomes	Total length of chromosomes (μm)	Total length of short arm (μm)	Form % (F%)	Chromosome characteristics
A	2	4.64	1.00	46.40	Two constrictions one in median and the other in the nearly sub-terminal in position.
C	14	16.96	7.80	45.99	
C	18	9.84	4.03	40.95	—do—
D	8	23.36	6.34	27.14	Nearly sub-terminal primary constriction.

Fig. 1. (a) Somatic metaphase chromosome $2n=48$ and (b) idiogram of *Xylocaprous granatum* Pop. Bhitarkanika.

chromosomes in the karyotype and the total chromosome volume of a karyotype was calculated by applying the formula $\pi r^2 h$, where 'r' and 'h' represent the radius and length of the chromosome, respectively. The form % (F%) of each chromosome was calculated following the method of Levan *et al.* (1964). Total form percentage of karyotype was the average of F% of a karyotype. Mean values of total genomic chromosome length and total chromosome volume with standard error were calculated.

Isolation of DNA

For DNA isolation, 5 g of leaf tissue were ground to fine power with liquid nitrogen and the power was suspended in 30 ml of suspension buffer (pH 8.0) containing 50 mM EDTA, 100 mM Tris-HCl, 0.8 M NaCl, 0.5 M sucrose, 2% Triton $\times 100$ and 0.1% β -mercapto-ethanol and incubated at 60°C for 30 min. The suspension was centrifuged at 10,000 g for 15 min at room temperature and the pellet was suspended in 20 ml of extraction buffer (20 mM EDTA, 100 mM Tris-HCl, 1.5 M NaCl, 2% CTAB and 1% β -mercaptoethanol, pH 8.0). Again the suspension was incubated at 60°C for 45 min followed by chloroform : isoamyle alcohol (24 : 1) extraction and ethanol precipitation at -20°C for 2 h.

Table 3. RAPD primers, their nucleotide sequence & number of RAPD bands generated from eight different populations of *X. granatum*

Primer	Primer Sequence (5'—3')	Total No. of bands	Pop-I	Pop-II	Pop-III	Pop-IV	Pop-V	Pop-VI	Pop-VII	Pop-VIII
			P(U)	P(U)	P(U)	P(U)	P(U)	P(U)	P(U)	P(U)
1. OPA 8	GTCACGTAGG	58	7(1)	6(0)	6(0)	7(0)	7(0)	2(5)	3(0)	5(1)
2. OPA11	CAATCGCCGT	45	5(1)	5(0)	5(0)	4(1)	4(0)	4(2)	3(0)	3(0)
3. OPA14	TCTGTGCTGG	41	3(0)	3(1)	3(1)	2(1)	2(1)	5(3)	4(0)	4(0)
4. OPD 8	GTGTCCCCCA	46	5(1)	5(1)	5(0)	4(0)	4(1)	4(1)	3(1)	3(0)
5. OPN 4	GACCGACCCA	41	2(1)	2(0)	4(0)	4(1)	4(1)	3(2)	3(2)	3(1)
6. OPD 2	GGACCCAACC	49	5(0)	4(2)	4(1)	3(1)	3(1)	4(2)	5(1)	5(0)
7. OPD12	CACCGTATCC	51	4(0)	5(0)	5(0)	6(0)	6(0)	2(4)	4(0)	7(0)
8. OPN11	TCGCCGCAAA	30	2(0)	2(0)	4(0)	4(0)	3(0)	0(3)	2(0)	2(0)
9. OPN15	CAGCGACTGT	39	3(1)	2(1)	3(1)	4(0)	4(0)	4(2)	3(0)	3(0)
10. OPA5	AGGGGTCTTG	44	4(0)	4(1)	3(2)	3(1)	4(0)	5(1)	3(1)	3(1)
Total 10 primers		444	40(5)	38(6)	42(5)	41(5)	41(4)	33(25)	33(5)	38(3)

Pop-population, P- number of polymorphic band, U-number of unique band.

Table 4. Similarities index among the eight populations of *X. granatum* using RAPD markers

	Pop I	Pop II	Pop III	Pop IV	Pop V	Pop VI	Pop VII	Pop VIII
Pop I	100							
Pop II	78.6	100						
Pop III	78.6	67.6	100					
Pop IV	80.5	69.4	93.5	100				
Pop V	83.4	68.1	86.7	91.3	100			
Pop VI	63.7	67.8	60.4	63.5	60.4	100		
Pop VII	61.6	72.8	47.2	47.6	48.5	42.6	100	
Pop VIII	69.3	85.3	63.8	63.7	54.4	60.4	78.4	100

DNA was hooked out and dried with vacuum drier and a very much less amount of TE (10 mM Tris-HCl, 1 mM EDTA) was added so as to dissolve the DNA. The DNA again purified treating with RNase at 37°C for 1 h followed by chloroform: isoamylalcohol extraction and ethanol precipitation in the presence of 0.3 M sodium acetate (pH 5.2). The DNA was spooled out, washed in 70% ethanol, air dried and dissolved in TE buffer and the DNA concentration was estimated in VersafluorTM Fluorometer (Bio-Rad, USA) using Hoechst 33258 as the fluorimetric dye. The DNA was diluted to final concentration of 25 ng μl^{-1} using TE buffer for using as template for RAPD analysis.

RAPD analysis

RAPD profiles were generated by using single decamer random oligonucleotide primers (Operon Technologies, Alameda, USA) in polymerase chain reaction (PCR) following the standard protocol of Williams *et al.* (1990). The sequence of primer is given in Table 2. Each polymerase chain reaction (PCR) amplification reaction mixture of 25 μl contained 25 ng of genomic template DNA, 200 mM of each dNTP, 25 ng of primer, 0.5 unit of Taq DNA Polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 10 $\times$ PCR assay buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl_2 , pH 9.0). The PCR reaction of the cocktail was carried out in a GeneAmpPCR 2400 thermal cycler (Perkin Elmer, USA) programmed for 45 cycles. The first cycle was programmed for 5 min at 94°C for denaturation, 1 min at 40°C for primer annealing and 2 min at 72°C for DNA polymerization. In the next 44 cycles the period of denaturation was maintained at 1 min while the primer annealing and DNA polymerization was same as in the first cycle. An additional cycle of 15 min at

Fig. 2. Amplification profiles of 25 accessions of eight populations of *X. granatum* using OPA-08 (a), OPD-12 (b) and OPN-11 (c); DNA marker (M) Gene Ruler 100 bp DNA ladder plus (MBI Fermentas, Lithuania). Pop-I=1, 2, 3 accessions, Pop-II=4, 5, 6, 7 accessions, Pop-III=8, 9, 10, 11 accessions, Pop-IV=12, 13, 14, 15, 16 accessions, Pop-V=17, 18, 19 accessions, Pop-VI=20, 21 accessions, Pop-VII=22, 23 accessions, Pop-VIII=24, 25 accessions).

Table 5. Genetic distance among the eight populations in *X. granatum* using RAPD markers

	Pop I	Pop II	Pop III	Pop IV	Pop V	Pop VI	Pop VII
Pop II	21.4						
Pop III	21.4	32.4					
Pop IV	19.5	30.6	6.5				
Pop V	16.6	31.9	13.3	8.7			
Pop VI	36.3	32.2	39.6	36.5	39.6		
Pop VII	38.4	27.2	52.8	52.4	51.5	57.4	
Pop VIII	30.7	14.7	36.2	36.3	45.6	39.6	21.6

72°C was used for primer extension. The amplified samples were stored at 4°C and separated by electrophoresis in 1.5 agarose gel in 1×TAE buffer for 3 h at 50 V. To determine the size of the polymorphic fragments. Gene Ruler 100 bp DNA ladder plus (MBI Fermentas, Lithuania) was used as size standard. DNA fragments were visualized by staining the gel with ethidium bromide and photographed for documentation.

RAPD data scoring and analysis

In RAPD analysis, the presence or absence of the bands were taken into consideration and the difference in the intensity of the band was ignored. From RAPD data a binary matrix was obtained. The matrix elaborated utilizing the multivariate analysis program NTSYS-pc (Rohlf 1993). The binary matrix was transformed in a similarity matrix using the Jacquard's similarity coefficient. The cluster analysis was carried out using the UPGMA (Unweighted pair group mean average) method (Sokal and Rohlf 1973).

Fig. 3. UPGMA dendrogram of eight population of *Xylocarpus granatum* obtained on the RAPD markers.

Results

Chromosome characteristics

Somatic chromosomes $2n=42$ was observed in all the populations of *Xylocarpus granatum*. The karyotype was symmetric in all the ecotypes with major variations of chromosome morphology. Chromosome length varied from $2.28\ \mu\text{m}$ to $3.25\ \mu\text{m}$; total chromosome length $54.80\ \mu\text{m}$ and total chromosome volume of $56.23\ \mu\text{m}^3$. The following types of chromosomes were found in the different populations of *X. granatum*;

Type A – Chromosomes are medium to small in size with two constrictions, one in nearly median and the other in nearly sub-terminal position.

Type C – Chromosomes are of small in size and having nearly sub-median constrictions.

Type D – Chromosomes are of small in size and having nearly sub-terminal chromosomes.

The detailed karyotypic analysis of a single population is presented in Table 2 (Fig. 1a) for understanding of the type of chromosomes (for other populations data not shown). In general variation of Type C and Type D chromosomes were observed among the populations. Since, the chromosome are very small in size not much of significant variations could be established to distinct the populations genetically.

RAPD analysis

Eight populations of *X. granatum* collected from eight different places showed some amount of DNA markers variations in population level. The number of amplification products ranged from 37 to 76 for eight different populations (Table 3) and polymorphism was between 36.25% to 72.5%. A total of 444 amplification products were observed of which was polymorphic (67.22%). RAPD profiles of eight different regions shared a number of common bands for all primers. The average number of amplification product obtained with one primer was 11.9% and 9.52% of the products were polymorphic. Population-VI showed a highest number polymorphic bands (58 bands out of 80 bands) generated by various primers showing a polymorphism of 72.5% (Tables 3 and 4, Figs. 2a, b, c). The profiles were very distinct out of the studied eight genotypes of different populations. RAPD profiles of eight populations with their representative accessions were amplified by different primers out of which OPA-8, OPD-12, and OPN-11 showed distinct DNA profile of each population without any significant variation between/among the plants collected under each populations (Figs. 2a, b, c).

In OPA-8, two high DNA markers of 3750 bp and 2630 bp were found to be unique to Pop-I,

Pop-III, Pop-IV and Pop-V in contrast to 3000 bp was not found in last two regions. The unique DNA bands of 2010 bp, 1270 bp, 925 bp, 380 bp and 230 bp were only obtained in Pop-VI-region while 1000 bp and 340 bp DNA fragments were confirmed to Pop-IV, Pop-V (Fig. 2a). DNA marker with 740 bp and 460 bp were reported in all populations except Pop-VI population while 506 bp was confirmed to population Pop-VI and Pop-VII. DNA band with 3300 bp, 2140 bp 1030 bp and 770 bp were unique to Pop-VI in OPD-12 primer. In that same primer DNA bands of 2900 bp, 1500 bp and 930 bp were found in all population besides Pop-VI. The DNA band with 1200 bp was found to be marker in Pop-II, Pop-VI and Pop-VIII whereas DNA band of 625 bp was a monomorphic band. DNA fragment, having 2530 bp was a good marker that was not found only in Pop-V, Pop-VI, Pop-VII with primer OPN-11 while 970 bp, 725 bp and 350 bp DNA fragment were found in all populations except Pop-VI. In Pop-VI, there were a number of unique bands of 2190 bp, 1770 bp and 490 bp (Fig. 2b). In OPN-4 primer, 1300 bp and 1064 bp DNA bands were found to be marker to Pop-III and Pop-V whereas 2200 bp DNA fragment was found to only Pop-IV and Pop-VII (Fig. 2c).

Cluster analysis

Pair wise comparisons were made for the RAPD profiles obtained from RAPD markers of eight populations of *X. granatum* showed a single tree with Pop-VI as a out group and rest are in a single cluster (Table 5, Fig. 3). Furthermore, that cluster again showed a out group of Pop-VIII, where the rest populations showed the cluster divided into two sub cluster confirm two distinct branch of the tree. Pop-VII and Pop-II showed closer affinity whereas pop-I, Pop-III showed relatively distinct relationships rather than the very close affinity of pop-IV and Pop-V through the use of 10 random primers in the representative samples of all eight populations.

Discussion

Chromosome number of all the 8 populations of *X. granatum* showed $2n=42$ chromosomes. Since the chromosomes were found very small, it was very difficult to find out any visual differences in the population level. As a result the RAPD marker analysis was made on all the populations and we could find some important observations and population specific bands that could distinguished Pop-VI as a genetically separate group of plants under the species *X. granatum*. The average number of amplification product obtained with one primer was 11.9% and 9.52% of the products were polymorphic. Pop-VI collected from Sundarbans of West Bengal showed a highest number of polymorphic bands (58 bands out of 80 bands) with 72.5% of polymorphism (Figs. 2a, b, c). The profiles were very distinct out of the studied eight genotypes of different populations. RAPD profiles of eight populations with their representative accessions were amplified by different primers out of which OPA-8, OPD-12, and OPN-11 showed distinct DNA profile of each population without any significant variation between/among the plants collected under each populations (Figs. 2a, b, c). This type of genetic variation in ecotype level was found in other mangrove associate like *Acanthus ilicifolius* (Jena and Das 2004).

The unique DNA bands of 2010 bp, 1270 bp, 925 bp, 380 bp and 230 bp were only obtained in Pop-VI-region while 1000 bp and 340 bp DNA fragments were the characteristics of Pop-IV, Pop-V (Fig. 2a). Distinct common DNA markers of 740 bp and 460 bp were reported in all populations except Pop-VI while 506 bp was confirmed to population Pop-VI and Pop-VII. DNA band with 3300 bp, 2140 bp 1030 bp and 770 bp were unique to Pop-VI in OPD-12 primer. In that same primer DNA bands of 2900 bp, 1500 bp and 930 bp were found in all population besides Pop-VI. In Pop-VI, there were a number of unique bands of 2190 bp, 1770 bp and 490 bp (Fig. 2b). In OPN-4 primer, 1300 bp and 1064 bp DNA bands were found to be marker to Pop-III and Pop-V whereas 2200 bp DNA fragment was found to only Pop-IV and Pop-VII (Fig. 2c).

Phylogenetic analysis of all the 8 populations showed a single tree with Pop-VI as a out group

and rest are in a single cluster. That confirms the significant genetic variability in Pop-VI. Pop-VII and Pop-II showed closer affinity whereas Pop-I, Pop-III showed relatively distinct relationships.

Mangroves exist in a constantly changing environment. Periodically the sea inundates the community with salty water while, at low tide, especially during periods of high rainfall, it may be exposed to floods of fresh water. Apart from suddenly altering the salinity levels, these fluctuations in water can alter temperatures as well. Mangroves are constantly subjected to physiological stress caused by fluctuating growing conditions (Chapeker 1994). Despite such extremes, they have successfully colonized suitable areas by developing morphological, physiological and reproductive adaptations (Clough *et al.* 1982, Clough 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 degrees of polymorphism. Present observations on *X. granatum* do support this presumption. This is because of its several and varied adaptational and distributional patterns. RAPD was analyzed on eight populations and the variability patterns were scored on the basis of amplified products. However the overall polymorphism in this species is of high magnitude. The observed inter-population divergence could be ascribed to the fluctuating microclimatic conditions in the regions where these genotypes inhabit. The analysis was carried out not only on a pair wise combination of variability but also accounted for the entire information given by all the genotypes against all the primers used.

The genetic resources of a species exist at two fundamental levels: i) genetic differences between individuals within a local population and ii) genetic differences between different local populations. Population genetics theory emphasized the evolutionary importance of the genetic structure of the species, that is, the distribution of genetic variation within and between populations. However, only in the last 15 years, through the electrophoretically detection of genetic variation at many protein loci, has it been practical to describe the amounts and distribution of genetic variation in natural populations (Nei 1975). Population structure is studied to answer a broad range of questions that can be study of ecotypic differentiation by investigating the influence of natural selection on population structure. Significant genetic differentiation is aimed to find between populations and specific fragments may be associated with soil types (Nevo *et al.* 1994, Dawson *et al.* 1993). Efforts to pressure genetic resources must take in to account the components of genetic variation, both within and between local populations.

The distribution of genotypes observed on the dendrogram is represented in Fig. 3. and shows that the RAPD data can be a powerful tool in assessing the genetic diversity. With regard to intra-site variability, genetic diversity within *X. granatum* collected from the same sites is very low. This is surprising because the species have evolved from the same habitats for a long period of time. The frequent inundation of seawater might have helped cross-pollination to account for this polymorphism. Though the dendrogram is quite revealing the groups are considered different with respect to sites. Two major components of genetic diversity within species is basically exist. Adaptive divergence *e.g.* that along gradients of selection is expected to result in phenotypic difference among population and does not require complete suppression of gene flow. By constant isolation is expected to result in phylogeographic structure at neutral molecular markers but does not necessarily result in phenotypic difference where both process operate, the resulting phenotypically and phylogenetically distinct populations can be recognized as distinct taxonomic entities (Avice and Ball 1990). Both molecular and phenotypic measures of divergence should be considered while measuring the diversity (Dizon *et al.* 1992, Vogler and Desalle 1994, Waples 1995). The evolutionary potential of individual populations can be predicted from their genetic makeup. The isolation combined with genetic drift/divergence selection will generate unique and irreplaceable combinations of genotypes, which may or may not be manifest as differences in phenotypes. This process of isolation and divergence was well studied here using molecular markers. In this case there is the significant phylogeographic structure. It is concluded that genetic diversity due to geographically isolation is not potentially re-

placeable. The genetic diversity within populations limits the response to selection especially in stressful or ecologically marginal environment.

Aknowledgements

We would like to acknowledge the Ministry of Environment, Government of India for supporting the financial assistance [Grant No. 3/7/2000-CS (M)] to carry out this work. We also acknowledge the help extended by Institute of Physics, Bhubaneswar, DST, Govt. of India providing liquid nitrogen free of cost.

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