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Full Length Research Paper

Inter-population variation of chromosome and RAPD markers of *Suaeda nudiflora* (Willd.) Moq. a mangrove species in India

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Inter-population genetic diversity in *Suaeda nudiflora*, a mangrove, was investigated through RAPD and chromosome analysis among five Indian populations. Somatic chromosome numbers 2n=36 reported for the first time in population (Pop) I and Pop-II whereas in Pop-III, 2n=36 and 40 chromosomes. Pop-IV showed 2n=54 numbers from high saline environment while Pop -V showed 2n=40 chromosomes. 102 RAPD bands were polymorphic out of a total 182 amplicons with an average of 3.64 bands per population per primer suggests genetic divergence in inter-population level. The dendogram based on the RAPD analysis showed two broad groups suggesting ecotypic adaptability in different saline habitat. The maximum overall relatedness of the pop-II with rest of four populations was 57% indicated by mean similarity while pop-IV revealed minimum mean similarity of 49% suggesting new cytotypes formation. The probable mechanism of overcoming high salinity stress by maintaining polyploidy (2n=54) is discussed.

Key words: Genetic divergence, genetic polymorphism, RAPD markers, somatic chromosome, *Suaeda nudiflora*.

INTRODUCTION

Mangroves have common needs to adapt to adverse environmental conditions associated with regular seawater inundation, for which individual species have developped different strategies and characteristics (Duke et al., 2002). Spatial patterns in genetically adaptive traits indicate that some populations survive more successfully under changing environment conditions (Dodd and Rafii 2002). Assessment of the genetic differences between populations of the same species gives a measure of the extent to which such populations are generally isolated from each other, while genetic comparison of different species shows information about the extent of divergence and potentially, when such divergence took place (Hogarth 1999). The extent of genetic difference among different populations is an important measure of the diversity of that area. Therefore, there is an obvious lack of knowledge concerning the genetic characteristics within and between populations of mangrove species as well as genetic relationship in mangrove ecosystems.

Suaeda nudiflora, a tropical halophytic grass that tolerates high temperature, generally found on the moist soil of river sea side ward fringe which always remain waterlogged with high and low tides. The plant species in the mangrove forests are constantly under environmental stress due to high saline conditions, extreme temperature and high salt deposition on the mud flat, therefore have adapted themselves to these frequent and fluctuating changes. *S. nudiflora* is dioecious with no vegetative propagation. Moreover, it is insect-pollinated; gene flow is expected to decrease considerably with distance. This unique plant, which has a versatile form with branches spreading on the soil surface, has a high food value for people along the coastal belt. However, under altered ecological and physical conditions in mangrove ecosystem, discernible changes were reported in genetic constitution of *S. nudiflora* (Tomlinson 1986, Jena et al., 2002) besides its morphology. Until recently, with exception of occasional reports on somatic chromosome analysis (Jena et al., 2002) most of the studies that investigate *S.*

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Table 1. Genotypes of *S. nudiflora* from different populations of Bhitarkanika mangrove forest, Orissa, India, with their physical characteristics and chromosome number.

Population	Source of collection	Physical Characteristics			Chromosome number (2n)
		Soil pH	Salinity (ppt)	Soil type	
Pop-I	Hansina Bhitargada	6.4-6.9	3-16	Clayey/fine sand	36
Pop-II	Chitta Kolha Rajnagar	6.3-7.0	5-18	fine silt/clay	36
Pop-III	Dangamal Righagarh	6.8-7.5	7-20	clayey, compact	40,36
Pop-IV	Gupti	6.9-7.8	10-23	Sandy and clayey	54
Pop-V	Talchua	6.7-7.6	6-21	Dark-clay soil	40

nudiflora used morphological characteristics of mangroves.

Molecular markers, unlike morphological markers, are stable and have been found to be very useful in population studies (Aitkin et al., 1994) they have been used to quantify accurately the extent of genetic diversity within and between population (Chalmers et al., 1992, Waugh and Powell 1992). The application of these markers in assessing intra-specific variation in mangrove species have been recently studied (Parani et al., 1997, Lakshmi et al., 1997). Among the various DNA marker systems, RAPDs have been used extensively for a variety of purposes, including population studies (Powell 1992). It, in particular, has found widespread application due to their technical simplicity and the availability of large numbers of arbitrary primers that saturate the genome.

In this study, five populations from different ecotypes of *S. nudiflora* growing in Bhitarkanika forest in India were genetically investigated. To examine the level of genetic variation of their diversity, chromosome analysis and RAPD markers were used. To our knowledge, this is the first genetic investigation done on *S. nudiflora*

MATERIALS AND METHODS

Plant Material

Five different populations (thirty individuals per populations) of *Suaeda nudiflora* (Willd) Moq. were collected from Bhitarkaniaka mangrove forest of eastern coast of India (latitude and longitude of 20^0 40'N, 86^0 52'E) for the present study. Bhitarkanika is a single compact patch of estuarine forest in Orissa about 192.9 sq. Km and is the second largest compact patch after Sundarbans of West Bengal in main land of India that is crossed by countless creeks, rivers and waterways. The physical characteristics of the study sites are given in Table 1. From each study site, root tips and young leaves were collected for chromosome and DNA isolation respectively. Roots were fixed and preserved in 70% ethanol for further investigation and young leaves were stored in a -85^0C.

Chromosome Preparation

Root tips were collected in the field and put in 0.05M oxiquoline solution and left at room temperature for 3 hrs and subsequently fixed in 1:3 ratio (acetic acid : ethanol) for over night then soaked in 45% acetic acid for 20 min stained in 2% acetic-orcine:1NHCl (9:1) for over night. A minimum of 10 roots from different plant/ population were squashed in 45% acetic acid and observed under microscope for chromosome count and photography.

Isolation of DNA

5 g of young leaf tissue was ground under liquid nitrogen and suspended in 20 ml of CTAB buffer (2% Cetyl Trimethyl Ammonium Bromide, 100 Mm Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl and 1% β-mercaptoethanol). The suspension was incubated at 60^0C for 30 min. The DNA was extracted in chloroform- isoamyl alcohol (49:1) and centrifuged at 5000 g for 20 min. The aqueous phase was taken up and DNA was precipitated with two volumes of chilled ethanol. The DNA was hooked out and dried with vacuum concentrator and a trace amount of TE buffer (10 mM Tris-HCl + 1mM EDTA, pH 8.0) was added to dissolve the DNA. The DNA again purified then treated with RNase at 37^OC for 1h followed by chloroform: isoamyl alcohol 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. DNA concentration was estimated using Versafluor TM Fluorometer (Bio-Rad, USA) using Hoechst 33258 as the fluorometric dye. The DNA was diluted to final concentration of 25ng μl^{-1} using TE buffer and used as template DNA for RAPD analysis. Leaves were collected and bulked from different plants for each population and replicated three times for DNA isolation.

PCR-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). Primer sequence is shown in Table 2. Amplification reaction mixture of 25 μl for each polymerase chain reaction (PCR) contained 25 ng of genomic template DNA, 200μM of each dNTP, 25ng of primer, 0.5 unit of Taq DNA Polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 10x PCR assay buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, pH 9.0). The reaction mixture was carried out in a Gene AmpPCR 2400 thermal cycler (Perkin Elmer, USA) in the following temperature cycles: holding at 94^0C for 5 min at start, followed by 44 cycles of 92^0C for 1 min, 40^0C for 1 min and 72^0C for 2 min and a final additional extension at 72^0C for 15 min. PCR- products were stored at 4^0C and electrophoretically separated in 1.5% agarose gel in 1×TAE buffer and visualized by ehidium bromide staining. To determine the size of the polymorphic fragments, Gene Ruler 100 bp DNA ladder plus (MBI Fermantas, Lithuania) was used as size standard. The gel was visualized under UV light and photographed for documentation.

RAPD Data Scoring and Analysis

In RAPD analysis, the presence or absence of the bands was taken into consideration and the difference in the intensity of the band was ignored. For all populations, bands on RAPD gels were scored as (1) when present or (0) when absent. Jaccard's similarity coefficient values (Jaccard, 1998) were calculated for each pair wise comparison between genotypes and similarity matrix was constru-

Table 2. RAPD primers, their nucleotide sequence and number of RAPD bands generated from five different populations of *S. nudiflora* of Bhitarkaniaka

Primer	Primer Sequence (5'--3')	Total No. of band	Pop-I		Pop-II		Pop-III		Pop-IV		Pop-V	
			P	U	P	U	P	U	P	U	P	U
1. OPA-05	AGGGGTCTTG	16	2	0	3	0	3	0	2	0	1	0
2. OPA-08	GTCACGTAGG	22	1	2	1	0	2	0	2	1	2	1
3. OPA-11	CAATCGCCGT	19	0	0	0	0	3	1	2	0	2	1
4. OPA-14	TCTGTGCTGG	17	1	0	1	0	2	0	0	1	2	0
5. OPD-02	GGACCCAACC	13	1	0	0	0	1	0	2	0	3	1
6. OPD-08	GTGTCCCCCA	27	2	0	3	0	2	0	4	2	4	0
7. OPD-12	CACCGTATCC	15	3	0	2	0	2	1	1	0	1	0
8. OPN-04	GACCGACCCA	24	0	0	0	0	3	0	2	0	4	0
9. OPN-11	TCGCCGCAAA	14	2	0	1	0	2	0	3	0	1	0
10.OPN-15	CAGCGACTGT	15	1	0	2	0	3	0	2	0	2	0

Pop-Popualation, P-Number of polymorphic band, U-Number of unique band.

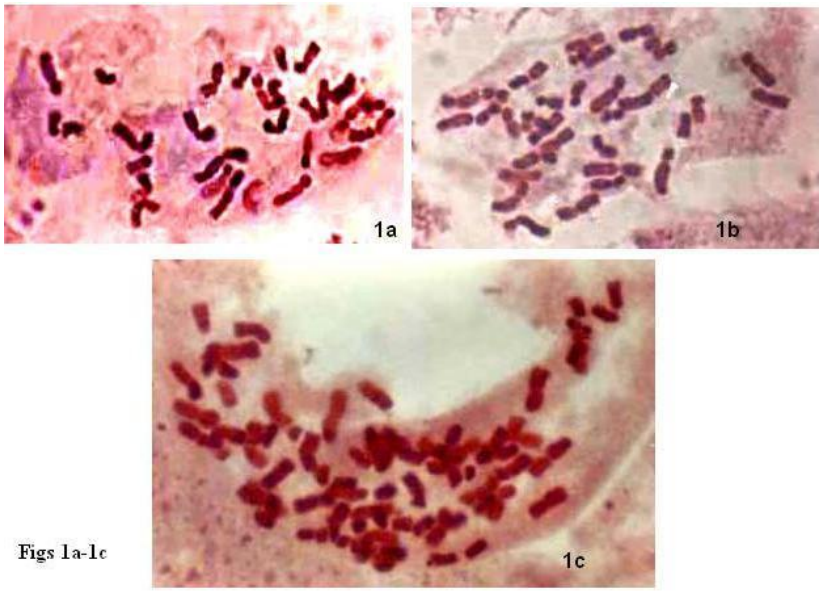


Figure 1a-1c. Somatic chromosome numbers in different population of *S. nudiflora* collected from Bhitarkanika mangrove forest of Orissa. Somatic cell of Pop-I showing 2n=36 (1a) collected from Hansia bhatighar having salinity 3-16 ppm, Pop-V showing 2n=40 (1b) chromosomes collected from Talchua area having salinity 7-20ppm, Pop-IV with 2n=54 (1c.) collected from Gupti with salinity 10-23 ppm.

ted. This matrix was subjected to unweighted pair group method for arithmetic average analysis (UPGMA) to generate a dendrogram using average linkage procedure. NTSYS-pc software (Rohlf, 1993) was used for analysis.

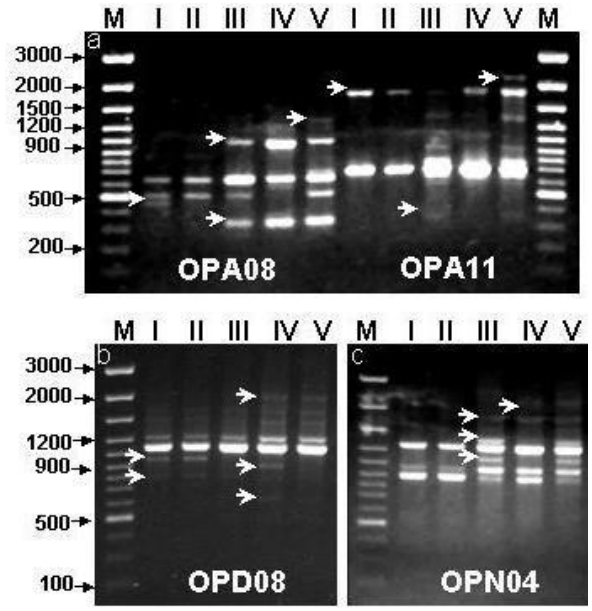
RESULT

Chromosome Analysis

Somatic chromosomes that were counted from all the collected populations from different saline zones showed very interesting results. The only population (Pop-IV) grown in Gupti having high salinity showed 2n=54 chro-mosomes (Table 1). The other two Pop-I and Pop-II showed 2n=36 chromosomes and the rest Pop-III showed 2n=36 mixed with 2n=40 chromosomes (Table 1, Figures 1a- c). However, the cells bearing 36 chromo-somes are not statistically significant. We attempted meiotic study in these population for better understanding of chromosome pairing behavior and natural ploidy form-ation were unsuccessful because of its very short dura-tion of metaphase period in the field condition.

RAPD Analysis

The number of amplification products ranged from 13 to 25 for different populations and polymorphism ranged between 48% to 61%. A total of 182 amplification products were observed out of which 102 (56%) were polymorphic. RAPD profiles of five populations shared a number of common bands for all primers. The average number of amplification product obtained with one primer was 18.2% and 56.04% of the products were polymorph-hic. Population specific polymorphic bands varied from 25 in Pop-III and Pop-V to 13 in Pop-II (Table 2). Where as percentage of polymorphism ranged from 48.38% in Pop-I to 52% in Pop-II and 55.55% in Pop-V to 60%-97% in Pop-IV. RAPD profile of five populations showed varia-tions in banding pattern when amplified by OPA-08, OPA-11, OPA-14 and OPN-04 separately (Figures 2a-c). In OPA-08, two DNA markers of 1344bp and 709bp were found to be unique to Pop-V in contrast to 453bp and 450bp unique bands to Pop-I while 1000bp and 340bp DNA fragments were observed in Pop-IV and Pop-V respectively. DNA marker with 2410bp was found a unique band to Pop-V while 1000bp DNA band was unique to Pop-III in OPA-11. For the primer, two DNA bands of 820bp and 330bp in Pop-III and Pop-V were also noted. The DNA bands with 1420bp were found as marker band in Pop-III, Pop-IV, and Pop-V whereas DNA band of 1120bp was found as marker in Pop-III and Pop-IV. DNA fragment, having 910bp was a good marker which was not only present in Pop-IV using OPA-14 primer while 300bp DNA fragment was unique to Pop-IV. In addition, in Pop-IV showed two marker bands



Figs. 2a-2c.

Figure 2. RAPD amplification profiles of five populations of *S. nudiflora* using OPA-8 & OPA-11(a), OPD-8 (b) and OPN-4 (c) and marker DNA (M) Gene Ruler 100bp DNA ladder plus (MBI Fermantas, Lithuania) from left to right showing major marker RAPD fragments i.e. 450bp in Pop-I, 300bp and 1000bp in Pop-III and 1344bp in Pop-V amplified by OPA-8 primers (arrow heads). OPA-11 primer produced marker bands of 330bp, 2134bp and 2410bp in Pop-II, Pop-I and Pop-V respectively. OPD-8 primers showed 850bp and 976b in Pop-I, 630, 980, 2400 bp in Pop-IV and OPN-4 amplified 1064bp, 1300bp, 1730bp in Pop-III and 1820 bp in Pop-IV (arrow heads).

of 630bp and 908bp in OPD-08 primer. In the same primer, 2000bp, 1650bp and 1200bp DNA fragment were found markers bands in Pop-IV and Pop-V while DNA marker of 976bp was found in Pop-I and Pop-II in contrast to 1233bp DNA marker in Pop-I, Pop-II and Pop-III. With OPN-04 primer, 1300bp and 1064bp DNA bands were found as marker to Pop-III and Pop-V whereas 2200bp DNA fragment was found to only Pop-IV and Pop-V. Three populations (Pop-III, Pop-IV, Pop-V) had a common DNA marker band of 1730bp. The maximum genotype-specific polymorphism (62.56%) was found in Pop-V followed by Pop-III (50%) and Pop-IV (37.5%) while in Pop-II and Pop-I there was the minimum number of polymorphic bands (4 and 6 respectively). The genetic distance was maximum between Pop-I and Pop-IV (Table 3).

Cluster analysis

Pair wise comparisons were made for the RAPD profiles obtained through the use of 10 random primers in the representative samples of all five genotypes of different populations of Bhitarakanika. The DNA characteristics of

Table 3. Genetic distance between the investigated population of *S. nudiflora* using RAPD analysis.

Pop-I	Pop –II	Pop- III	Pop-IV	Pop-V	
Pop- I	100				
Pop- II	16.6	100			
Pop- III	50.0	42.4	100		
Pop- IV	63.4	57.2	46.9	100	
Pop- V	61.3	55.2	35.5	38.8	100

all the populations of *S. nudiflora* showed a single tree with major two branches. Pop-I and Pop-II clustered together with a similarity coefficient of 0.83 and made one branch of the tree while the rest three population formed the other branch of the tree where pop-III and pop-V are sister group. Among these three population, Pop-III and Pop-V showed a closer affinity with 64% of similarity while the Pop-IV showed a very less close similarity with Pop-III (53%) and Pop-V (61%). The highest value of mean similarity coefficient 0.57 was found in Pop-II, followed by Pop-III (0.56). The lowest value of mean similarity coefficient was recorded for the genotype collected from Pop-IV.

DISCUSSION

A DNA based diagnostic assay like RAPD is able to identify genotypes directly and can therefore help mitigate complications arising from earlier cytological and morphological studies. The utility of combined previous cytogenetics studies (Jena et al., 2002) and RAPD markers in resolving phylogenetic patterns in *S. nudiflora* is

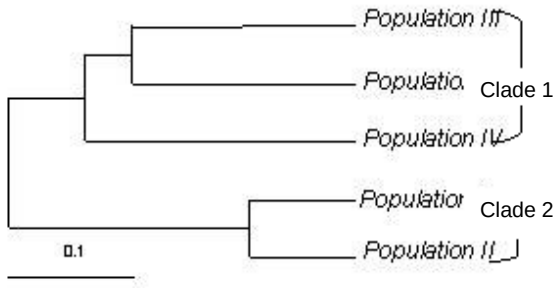


Figure 3. UPMGA phenogram of five populations of *Suaeda nudiflora* from five different habitats of Bhitarkanika mangrove forest of Orissa, India.

clearly demonstrable. Rapid genetic differentiation is likely among population groups with divergent chromosomes . Between adjacent geographically defined cytotypes of *S. nudiflora*, there was a significant polymorphism. Remarkably high individual gene diversity has been observed between populations as evedinced by chromosome records in different populations of *S. nudiflora* (Figures 1a-c). Since 1930, investigators have tried to associate the numerical chromosome variation found in plants with the environment and to relate the different cytotypes occupying different niches in terms of temperature, luminosity, humidity etc. (Bennet 1987). Intra-specific chromosome variation was extensive and informative to conservation biologists. Although we have assumed earlier the existence of different cytotypes of this species (Jena et al., 2002), it has been now better understood with chromosomal and RAPD data. RAPD data support the existence to defined cytotypes for adaptation of different populations at various environmental conditions. Gene diversity between populations was more prominent in the gel figures, where each genotype from each population has been amplified with the same primer. In addition to population, cytogenetic data and RAPD data at inter-population levels have proved to be extremely instructive in developing a better understanding of divergence. In particular, there are highlighted distinct populations and local groups within same species, which are not only genetically distinct but are confined to geographically restricted and unique plant communities. In the present study we showed the variability of the RAPD banding pattern in *S.nudiflora*, which was evident by chromosome number, although morphologically they are not distinguishable. Our findings confirm genetic divergence among populations belonging to different biological units. Such molecular techniques are useful even to delimit species, especially morphologically similar taxa. Ecological (habitat) comparison is also powerful method to recognize different biological units with similar morphology, especially when they are sympatrically distributed. The observed inter-population divergence for populations with different geo-location could be attributed to the adaptability with the fluctuating micro-climatic conditions with different degrees of temperature, light tolerance, and salinity gradient (Dawson et al., 1993).

UPGMA dendogram (Figure 3) shows too clear clades, where pop-III, pop-V and pop-IV are grouped together (clade1), whereas pop-I and pop-II are grouped in clade 2. Among populations, genetic variations are relatively high suggesting that the populations are largely isolated from each other with gene flow. Local selection and restricted gene flow between the genotypes has been contributed more to the limited genetic variability of this species. Thus, it seems likely that fragmentary process will accelerate in this species, which appear to be an inherently slow group to respond in an evolutionary sense. Since the genotypes were physically isolated, the genetic content of the individuals that originally colonized the locations might be one of the causes of divergence.

In conclusion, it is observed that though *S. nudiflora* does not show significant morphological variations, the present investigation using both chromosomal data and

molecular markers reveals that substantial inter-population variation does exist that confirm the existence of cytotypes and genotypes for better adaptation of this species in ecotype level in different magnitude of adverse condition in Bhitarakanika mangrove forest, Orissa, India.

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