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Variation in karyotype and DNA markers in different ecotypes of a mangrove associate, *Suaeda nudiflora* (Willd.) Moq. from Bhitarkanika, Odisha

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ABSTRACT

Karyotype and chromosome number variation was observed in different ecotypes of *Suaeda nudiflora*, a mangrove associate collected from Bhitarkanika mangrove forest of Odisha, India. The chromosome number varied from 2n=36 to 54 and Ecopye IV from Gupti (2n=54) and Ecotype V from Ekakula (2n=40) with changes in karyotype was observed beside the normal diploid number 2n=23 in Ecotype-I (Dhamra), -II (Rajnagar) and –III (Dangmal). Investigation of RAPD profile among these ecotypes revealed genetic variation among the ecotypes. Some of the ecotype specific bands are obtained in Ecotype-IV like 100 bp in OPD-12, 300bp in OPN15 and 1000bp in OPA05 are unique which can be used for developing SCAR markers for future use. Ecotype-V having 400bp in OPN-15, 500bp in OPA-14, 800bp in OPA-05 are found marker bands. While some of the monomorphic bands are common in all the ecotypes like 500pb in OPA-05 beside the marker bands of 600 bp and 1500 bp in OPA-14 in Ecotype-I are characteristics of the Ecotypes. Phylogenetic relationship and chromosome number and karyotype suggest the possible link of saline adaptability with ploidy changes of different ecotypes and subsequent changes in DNA profile rather than its epigenetic modifications in DNA level.

1. Introduction

Mangroves are one of the most threatened ecosystems all over the world today due to direct and anthropogenic indirect degradation (Alongi, 2002; Duke *et al.,* 2007) and has resulted in great loss of genetic diversity in the mangrove ecosystem (Maguire *et al.,* 2002; Triest *et al.,* 2008). Conservation of mangrove including genetic resources implicates not only to protect the coastal areas and communities from seawater intrusion and potential changes in sea level rise but also to ensure the availability of resources for future use through adaptation to changing environments. Information on genetic diversity of mangrove species is very important in planning for conservation of genetic resources and afforestation program (Hamrick *et al.,* 1992; Duke *et al.,* 1998). Knowledge of genetic diversity and its causes can provide insight into their ecological and evolutionary histories; thus, such information also may help in conservation and restoration. The genetic variation of a species can be assessed by different techniques from morphological and metric characters in the field to biochemical and molecular markers in the laboratory (Graudal *et al.,* 1997). Molecular markers are important tools for identifying appropriate population sources for reforestation of these unique and important habitats of mangrove forests (Schwarzbach and Ricklefs, 2001) beside their chromosome status.

Suaeda nudiflora, a tropical halophytic mangrove associate commonly known as 'Giringa' leafy vegetable that tolerates high temperature which is found on sea ward fringe which always remain waterlogged with high and low tides. This unique plant, which has a versatile form with branches spreading on the soil surface. It is very much important for its food value used by poor man in the coastal belt. Due to the hostile condition of the mangrove environment, the plant species in the mangrove forests are

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constantly under environmental stress due to high saline conditions, extreme temperature and high salt deposition on the mud flat and have adapted themselves against these frequent and fluctuating environmental changes. *Saudea* is dioeciously plant that lacks vegetative propagation. Moreover, the species is insect-pollinated and thus gene flow is expected to decrease considerably with distance. However, under altered ecological and physical conditions in mangrove ecosystem, discernible changes were reported in genetic architecture of *Suaeda nudiflora* (Jena and Das, 2006) besides its morphology (Tomlinson, 1986). The chromosome number were reported to be 2n=36 in *S. nudiflora* (Kumar and Subramanian, 1988; Jena *et al.,* 2002). Molecular markers, such as allozymes, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites (SSR), Inter-simple sequence repeats (ISSR), and DNA sequence, have proven to be a very efficient means to investigate population genetics of mangrove species (Triest *et al.,* 2008). The application of these markers in assessing intra-specific variations in mangrove species have been recently studied (Parani *et al.,* 1997; Lakshmi *et al.,* 1997). Among the various DNA marker, RAPDs have been used extensively for a variety of purposes, including ecotype studies. Although several studies on population genetic in mangoves have been reported (Nunez-Farfan *et al.,* 2002; Arbelaez *et al.*, 2007; Pil *et al.,* 2011), the extent and patterns of genetic diversity in this mangrove species remain obscure. Genetic diversity is very much critical for adaptation to environmental changes and for long-term survival of the species. The genetic variations have to be conserved before completely restoring the ecology role that has long been lost due to the mangrove ecosystem deforestation (Schwarzbach and Ricklefs, 2001). The objective of the present study was to assess the genetic variation among different ecotypes grown in various saline regime with a chromosome number, detail karyotype and RAPD profile of *S. nudiflora* distributed in East coast of India besides our earlier report with ploidy changes (Jena and Das, 2006) to acquire useful genetic information to support mangrove forest conservation.

2. Material and Methods

2.1 Plant Materials

Different ecotypes of *Suaeda nudiflora* from Bhitarkaniaka mangrove forest of Odisha, India with a latitude and longitude of 20^0 40'N, 86^0 52'E respectively were collected for the present study (Table 1). From each study site, root tips and young leaves were collected for chromosome number and detail karyotype study and DNA isolation respectively. Young leaves were stored in a –85^0C freezer for not more than two weeks before DNA extraction and roots were subjected to pretreatment and fixation.

2.2 Chromosome preparation and karyotype

Root tips were collected in the field and put them in 0.05M oxiquoline solution and kept in room temperature for 3 h and subsequently fixed in 1:3 (acetic acid: ethanol) for over night. Root-tips were transferred to 70% ethanol in the field and kept for further study. Preserved root tips were soaked in 45% acetic acid for 20 min stained in 2% acetic-orcine:1NHCl (9:1) for over night. Root tip squash was made separately for each ecotypes in 45% acetic acid and observed under microscope for chromosome count and photography. For karyotype study, each ecotypes at least 5 roots from different plants were taken following the procedure of (Das and Mallick, 1993a).

2.3 DNA isolation

Total genomic DNA was extracted from leaf tissues using modified cetyl trimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle, 1987). 5g of each of young leaf tissue was ground under liquid nitrogen and suspended in 10 ml of CTAB buffer (2% Cetyl Trimethyl Ammonium Bromide, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4M NaCl and 1% b-mercaptoethanol). The suspension was incubated at 60^0C for 1h. The DNA was extracted in chloroform:isoamyl alcohol (24:1) for 10 min with gentle shaking and centrifuged at 10,000g for 20 min. The aqueous phase was taken in a separate clean sterilized tube and DNA was precipitated with two volumes of chilled iso-propanol. The DNA was hooked out and dried with vacuum concentrator and dissolved in TE (10 mM Tris-HCl + 1mM EDTA, pH 8.0). The DNA again purified treating with RNase at 37^oC for 1h followed by phenol: chloroform:isoamyl alcohol extraction (25:24:1) followed by chloroform:isoamyl alcohol (24:1) and centrifuges. The supernatant was precipitation with chilled ethanol in presence of 0.3M 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 Versafluor TM Fluorometer (Bio-Rad, USA) using Hoechst 33258 as the flurometric dye. The quality of the DNA was also evaluated using 1% agarose gels and then quantified by UV-Spectrophotometer (Shimadzu, Kyoto, Japan). The DNA was diluted to final concentration of 25ng ml^{-1} using TE buffer and used as template DNA for RAPD analysis. The material for PCR analysis were stored at -20 ^{0}C.

2.4 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. Amplification reaction mixture of 25ml for each polymerase chain reaction (PCR) contained 25ng of genomic template DNA, 200mM of each dNTP, 25ng of primer, 0.5 unit of Taq DNA Polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 10 × PCR assay buffer (50mM KCl, 10m M Tris-HCl, 1.5mM $MgCl_2$, 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 5min at start, followed by 44 cycles of 92^0C for 1 min , 40^0C for 1min and 72^0C for 2min and a final additional extension at 72^0C for 15min. The amplified samples were stored at 4^0C and electrophoretically separated in 1.5% agarose gel in 1×TAE buffer and visualized by ethidium bromide staining. To determine the size of the polymorphic fragments, Gene Ruler 100bp DNA ladder plus (MBI Fermantas, Lithuania) was used as size standard. The gel was photographed under UV light for documentation.

2.5 RAPD data scoring and statistical 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 ecotypes, bands on RAPD gels were scored, as present (1) or absent (0). Jaccard's similarity coefficient values (Jaccard, 1998) were calculated for each pair wise comparison between ecotypes and similarity matrix was constructed. This matrix was subjected to unweighted pair group method for arithmetic average analysis (UPGMA) to generate a dendrogram using average linkage procedure. All computing were carried out using NTSYS-pc (Rohlf, 1993). The RAPD data were further subjected to analysis of molecular varience (AMOVA) as described by Excoffier *et al.* (1992) using three hierarchical levels: individual, population and their regions with the GenALEX software (Peakall and Smouse, 2001), and also used for principal coordinate analysis (PCA) of the relationship between the distance matrix and elements based on the first two principal coordinates.

3. Result

3.1 Chromosome and karyotype analysis

Somatic chromosomes were counted from all the ecotypes collected from different saline zones showed chromosome number variation from 2n=36 to 54. Ecotype-I, II and III showed 2n=36 chromosome while Ecotype-IV with 2n=54 and Ecotype-V with 2n=40 were recorded (Table 1, Figs 1a-1c). On the basis of the size and position of the constrictions on the chromosome, a number of chromosome type were found to be common within the ecotype studied, though there were minute differences of the karyotype. A general description of the representative types of chromosomes is given bellow.

Type A: Medium sized chromosome with primary and secondary constrictions at sub-medium and subterminal in position respectively.

Type B: Medium sized chromosome with two constrictions one in the median to sub-median position and other in the sub-terminal position.

Type C: Medium to small sized chromosome with median primary constrictions.

Type D: Medium to small sized chromosome with sub-median primary constrictions.

There are no much of variation in karyotype formula of Ecotype-I,II and III was found with about same number of median and sub-median chromosomes. Ecotype-IV showed 4 each of Type A and Type B chromosomes with secondary constrictions with 33 number of medina chromosome (Type C) and 15 number of sub-median chromosome (Type D). However, Ecotype-IV showed less numbers of sub-median chromosomes (Type D) as compared to the number of median chromosome (Table1, Fig.1).

Table 1
Ecotypes of *S. nudiflora* from different places of Bhitarkanika, Odisha, India, with their somatic chromosomenumber and karyotype.

Ecotypes	Place of Collection	Soil type	pH	2n	Karyotype formula
I	Dhamara	Heavy clayey	6.2-6.5	36	2B+18C+16C
II	Rajnagar	Silt mixed with clay	6.5-7.0	36	2B+18C+16C
III	Dangamal forest	Heavy silt clay	5.5-7.2	36	2B+18C+16C
IV	Gupti	Sandy silt	6.8-7.6	54	4A+4B+33C+15D
V	Ekakula	Sandy	6.5-7.3	40	4A+4B+24C+8D

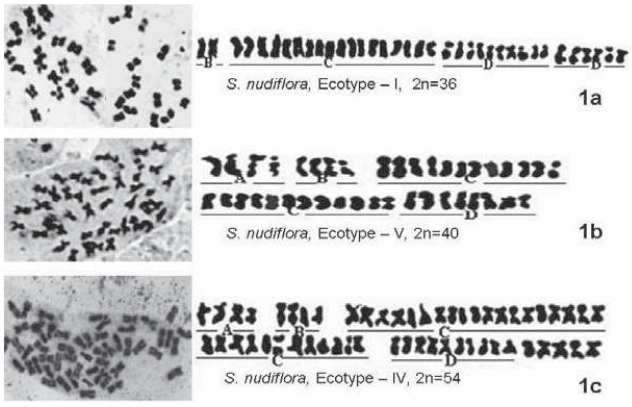

Fig. 1. Somatic chromosome numbers with corresponding karyotypes of different respective of *S. nudiflora* collected from Bhitarkanika forest mangrove of Orissa. Ecotype-I showing 2n=36 (1a), Ecotype-V showing 2n=40 (1b), Ecotype-IV with 2n=54.

3.2 RAPD analysis

The agarose gel electrophoresis derived PCR amplification photographs were analyzed and it was found that a total of 313 amplicons were amplified using 14 Operan primers. The fragment size varied from 100-2800bp and the lowest range (100-950bp) was observed OPA-10 primer. The total polymorphic percentage was 49.20% were as it varied among the Ecotypes ranging from 6.389% (Ecotype-I) to 15.015% (Ecotype-IV). The number of amplification products ranged from 20 to 35 for different ecotypes. RAPD profiles of five ecotypes shared a number of common bands for all primers. Ecotype specific polymorphic bands varied from 2 to 5 among the ecotype (Table 2). RAPD profile of five ecotypes showed variations in banding pattern when amplified by OPA-05 with a prominent marker band of ~1000bp and ~800bp found unique in Ecotype-IV and Ecotype-V respectively. OPA-14 although produced major monomorphic bands while succeed to produced two unique bands (1500bp and 600 bp) for Ecotype-I and 500bp for Ecotype-V (Figs. 2a & 2b). In OPD-12, a very low size fragment of ~100bp found as unique band for Ecotype-IV. Two marker bands of 300bp and 400bp were recorded in OPN-15 primer for Ecotype-IV and Ecotype-V respectively.

3.3 Cluster analysis

Pair wise comparisons were made for the RAPD profiles obtained through the use of 14 random primers in the representative samples of all five genotypes of different Ecotypes of Bhitarakanika. Ecotype-I. Ecotype-II and Ecotype-II clustered together with a similarity coefficient of 0.83 made one branch while the rest two Ecotype i.e. Ecotype-

Table 2
RAPD profile generated from different ecotypes of *S. nudiflora* with percentage of polymorphism.

Primer	Primer Sequence	No. of Amplicon	Eco-I	Eco-II	Eco-III	Eco-IV	Eco-V	Size range
OPD-02	5'GGACCCAACC3'	18	1	0	1	2	3	300-1200
OPA-05	5'AGGGGTCTTG3'	27	3	5	3	2	3	200-1500
OPA-07	5'GAACGGGTG3'	16	3	1	2	2	3	300-1050
OPA-08	5'GTCACGTAGG3'	22	2	3	2	3	2	300-1600
OPA-10	5'GTGATCGCAG'	27	2	3	1	6	3	100-950
OPA-11	5'CAATCGCCGT3'	19	0	0	1	5	2	200-1650
OPA-13	5'CAGCACCCAC3'	28	2	1	1	4	2	230-1200
OPA-14	5'TCTGTGCTGG3'	30	1	1	2	6	2	150-2800
OPD-02	5'GGACCCAACC3'	19	2	2	4	5	5	350-1800
OPD-08	5'GTGTCCCCCA3'	27	2	1	2	4	4	300-2300
OPD-12	5'CACCGTATCC3'	25	1	2	2	1	1	200-2500
OPN-04	5'GACCGACCCA3'	24	0	0	3	2	2	350-980
OPN-11	5'TCGCCGCAAA3'	14	2	1	2	3	1	200-950
OPN-15	5'CAGCGACTGT3'	17	1	2	3	2	2	350-1200
	Total Bands	313	20	22	30	47	35	
	Polymorphic %	49.20	6.389	7.028	9.584	15.015	11.182	

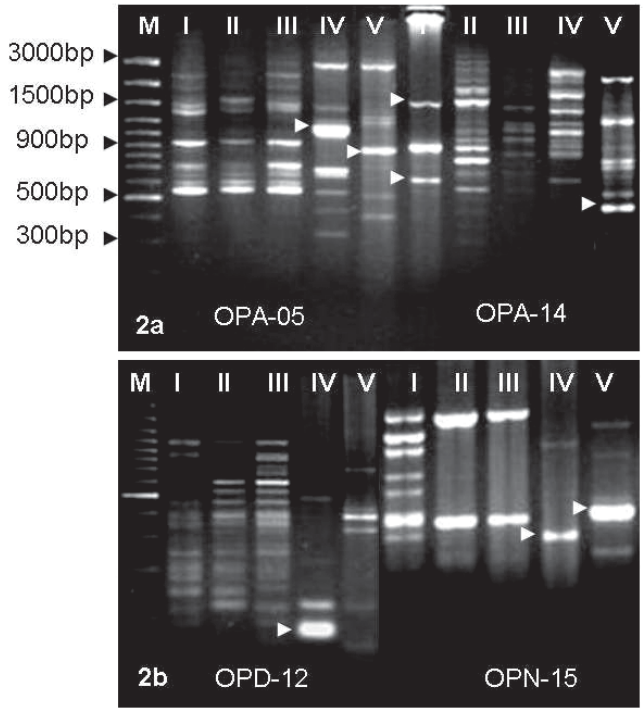

Fig. 2. RAPD amplification profiles of five ecotype of *S. nudiflora* using OPA-5 and OPA-14(2a), OPD-12 and OPN-15 (2b). M=Gene Ruler 100bp DNA ladder plus (MBI Fermantas, Lithuania), I to V = Ecotypes from left to right showing major marker RAPD fragments (arrow heads).

IV and Ecotype-V formed the other branch of the tree (Fig. 3). The highest value of mean similarity coefficient 0.57 was found in Ecotype-I and Ecotype-II followed by Ecotype-III (0.56). The lowest value of mean similarity coefficient was recorded in Ecotype-IV and Ecotype-V.

AMOVA helped to pertain the RAPD variations among different Ecotypes and among individuals within a ecotype. About 3.35% molecular variation within ecotypes and about

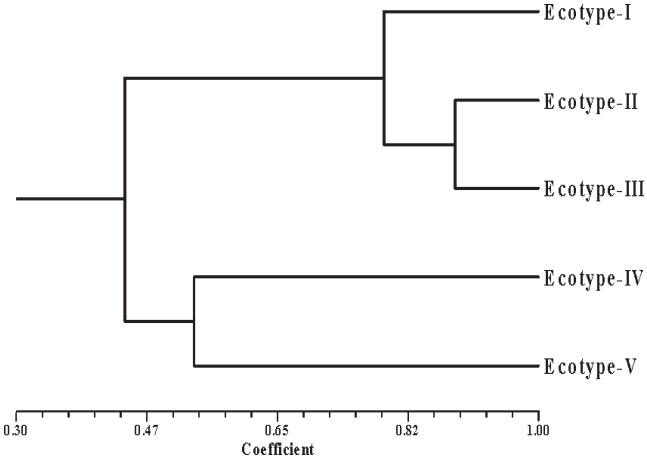

Fig. 3. Dendrogram showing genetic relationships among ecotypes of *S. nudiflora* on the basis of RAPD analysis.

~48% variation among Ecotypes were recorded. This may be useful in strategies for germplasm collection and evaluation. The PCA analysis (Fig. 4) was comparable with the cluster analysis (Fig. 3), with all similar chromosome number Ecotypes having 2n=36 chromosomes i.e. Ecotype-I, -II and –III cluster together from the rest of the Ecotypes. Gupti (Ecotype-IV) having 2n=54 and Ekakula (Ecotype-V) with 2n=40 were distinct from other genotypes in the PCA with a separate group all together.

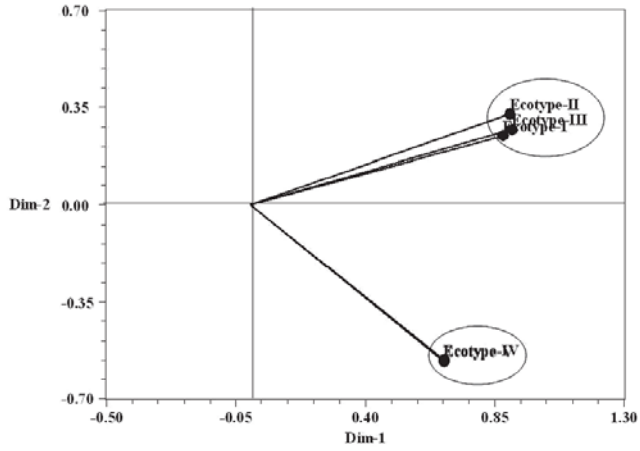

Fig. 4. Two-dimensional plot of principal component analysis of five Ecotypes (I-V) of *S. nudiflora*.

4. Discussion

Numerical variation of somatic chromosomes were recorded in ecotype level in *S. nudiflora*. The somatic chromosome 2n=36 which was found in Ecotype-I, II and III reconfirm the earlier report (Kumar and Subramanian, 1988; Jena *et al.,* 2002). However, the chromosome number for Ecotype-IV with 2n=54 from Gupti area of Bitarkanika which was confirmed earlier by us (Jena and Das, 2006). But a new Ecotype-V collected from Ekakula of Bitarkanika showed 2n=40 chromosome. Same type of cytotypes were also reported earlier from Talchua region of Bitarkaika (Jena and Das, 2006). We performed the detailed karyotype of all the Ecotypes and revealed that a high number of median constricted chromosomes as compared to sub-median chromosome were observed from Ecotype-IV which grown in high saline region of sanctuary (Table 1, Fig.1). Shifting of sub-median constricted chromosome to median chromosome in the karyotype of Ecotype-V was found which was collected from Ekakula Island of the mouth of the sea. The structural alteration of the chromosome morphology in Ecotype-IV and Ecotype-V of high saline zone might be due to partial duplication of chromosomes or translocation between the chromosomes with or without secondary constricted chromosomes during ecotype/cytotype formation for better adaptability of this plant in these hostile conditions (Das, 1991; Das and Mallick, 1993a,b; Das *et al.,* 1994).

DNA marker profile identify Ecotypes directly and therefore help to mitigate complications arising from earlier cytological and morphological studies. Between adjacent geographically defined Ecotypes of *S. nudiflora*, there was a significant polymorphism. Remarkably high individual genetic diversity was observed in Ecotype-IV and Ecotype-V with high chromosome number in high saline zone indicate the genetic changes in Ecotype level of *S. nudiflora* (Fig. 2). Since 1930, investigators have tried to associate the numerical chromosome variation found in plants with the environment and to relate the different cytotypes to the occupation of different niches in terms of temperature, luminosity, humidity etc. (Bennet, 1987). Although we have assumed earlier the existence of different cytotypes and population of *S. nudiflora* (Jena *et al.,* 2002; Jena and Das 2006) it has been now better understood with chromosomal, karyotype and RAPD data for the existence of new Ecotypes with high chromosome number in other locations of Bitarkanika like Ekakula which needs thorough investigation for further discovery of new cytotypes from this area for *S. nudiflora*. RAPD data support the existence to defined cytotypes for adaptation of different Ecotypes at various environmental conditions. RAPD profile of five ecotypes showed variations in banding pattern when amplified by OPA-05 with a prominent marker band of ~1000bp and ~800bp found unique in Ecotype-IV and Ecotype-V respectively. The marker bands of 600 bp and 1500bp in OPA-14 for Ecotype-I and 500bp for Ecotype-V might be due to genetic changes of the Ecotypes (Fig. 2). Gene diversity between Ecotypes was more prominent in the gel figures, where each genotype from each Ecotype has been amplified with the same primer. In addition to Ecotype, cytogenetic data and RAPD data at inter-ecotype levels have proved to be extremely instructive in developing a better understanding of divergence. In particular, there are highlighted differences between Ecotype and local groups within same species, which are not only genetically distinct but are confined to geographically restricted and unique plant communities. In OPD-12, a very low size fragment of ~100bp found as unique band for Ecotype-IV. Two marker bands of 300bp and 400bp were recorded in OPN-15 primer for Ecotype-IV and Ecotype-V respectively. In the present study we showed the variability of the RAPD banding pattern in *S. nudiflora,* which was evident by chromosome number and detail karyotype data with no much of morphological changes of the plant. Hence, it is suggested that there is a genetic divergence among the different ecotype as they found to belong to different biological units. Ecological comparison is also powerful method to recognize different biological units with similar morphology, especially when they are distributed. The observed inter-ecotypic divergence could be ascribed to the adaptability with the fluctuating micro-climatic conditions of different degree of temperature, light tolerance, salinity gradient for their different geo-locations (Dawson *et al.,* 1993). Examination of the UPGMA dendrogram (Fig. 3) clearly showed the isolated position of Ecotype-IV and Ecotype-V from a single cluster from the rest of the three Ecotypes i.e. Ecotype-I, Ecotype-II and Ecotype-III. The former Ecotypes have the higher chromosome number with an altered karyotype which might be a adaptive strategies for the mangrove associate to survive in high saline area with increase chromosome number with varied genetic makeup.

This is for the first time assessing a huge percentage of inter-ecotypic genetic variation of *S. nudiflora* through molecular genetic studies (RAPD). Between Ecotype, genetic variations are relatively high suggesting that the ecotype are largely isolated from each other with little mutual 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 Ecotype 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 chromosomal data RAPD data reveals that substantial inter-ecotype variation does exist that confirm the existence of cytotypes and genotypes for better adaptation of this species in high saline area

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