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Genetic Relationship Between Populations of *Suaeda maritima* (L) Dumort. Growing along the Coastal Belt of Purba Medinipur District, West Bengal, India in Respect of AFLP Performance

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A B S T R A C T

Suaeda maritima (L.) Dumort., a halophyte herb of the family Chenopodiaceae, grows gregariously along the coastal belt of Purba Medinipur district of West Bengal in India. The geographical location of this area is in between 21°63'16''N to 21°70'50''N latitude and 87°54'63''E to 88°12'40''E longitude. This annual salt marsh species is regularly used by the local people as food and also for curing many a disease. Earlier works report on phenotypic plasticity of it and other aspects of morpho-anatomical features as well as useful phytochemical elements of it. Since the populations of this species in this coast are not growing contiguously, in search of any plasticity due to genetic difference amongst the individuals of different populations AFLP analyses were carried out. The studied area was transected into eight zones. The genetic distances were calculated based on the AFLP bands that had been amplified using the 12 primer combinations. The similarity indices (SI) ranged from 0.19 to 0.83. The dendrogram was created using the UPGMA and the genotypes were found to be classified into 2 major groups. This study revealed the existence of genetic diversity even among the individuals of closely growing populations of this species, indicating significant dynamism in it, a fact which may be quite imperative of its ability to cope up with the fragile physical environment it grows in.

***Key words*:** *Suaeda maritima*, AFLP, Cluster analysis, Coastal belt

The genus *Suaeda* Forssk. ex J.F. Gmel., under the family Chenopodiaceae, comprises 110 species of halophytic herbs or shrubs distributed worldwide along the seashores [1-3]. Most of the species of *Suaeda* are generally found to grow along the coastal belts of tropical and sub-tropical regions between 25 N to 25 S latitude throughout the world [4-5]. They are annual, herbaceous, succulent bushy mangrove associate plants with preferably growing in the soils rich in salt [6-7]. *Suaeda maritima* (L) Dumort**.** is one of the most important species under the genus due to its use as vegetable and for curing different maladies [8] on having cardio-protective and hepato-protective properties due to presence of triterpenoid - alpha amyrin [9-11]. It grows along the coastal belts of Indian subcontinent [12] and so also grows luxuriantly in the coastal belt of Purba Medinipur district of West Bengal in India [13]. However, it does not grow contiguously on the said coastal belt, instead occurs as punctuated populations exhibiting the morphological and phyto-chemical variation amongst them [14]. In this parlance AFLP analysis of the sample individuals representing different populations of the species has been carried out in search of diversity.

Amplified fragment length polymorphism (AFLP) has been widely used for the identification, standardization, and determination of genetic diversity of plants [15-16]. Moreover, the marker can be used to survey a whole genome without any prior sequence knowledge [17]. AFLP markers have been used favourably to identify herbal plants and to evaluate genetic diversity in various species [18-19]. There are reports on molecular phylogenetic analysis of the subfamily Suaedoideae [20-22]. By using ribosomal DNA and chloroplast DNA markers researchers reflected a discordant relationship among few species of *Suaeda* [23-24]. The populations of *Suaeda maritima*, here understudy, show subtle phenotypic variation that might be having some genetic cause behind. AFLP among the samples of the populations has revealed the presence of subtle genetic variation.

MATERIALS AND METHODS

Plant materials

The fresh young leaves of *Suaeda maritima* (L.) Dumort. were collected from eight distantly situated locations of the study area at the coastal belt of Purba Medinipur district of West

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Bengal in India. The plant species were authenticated by the Central National Herbarium, Sibpur, India vide voucher specimen number CNH//2015/ Tech.II/17/299. The voucher specimens were deposited at the Herbarium of Vidyasagar University, India.

Genomic DNA extraction

5g of fresh young leaves of each sample were lyophilized (freeze dried) in a Virtis freeze drier for 24-36 hours to obtain completely dry samples. 50 mg of each lyophilized leaf samples were ground to fine powder. The genomic DNA was extracted from these leaf samples by CTAB based method [25]. DNA quantification was performed by subjecting Agarose gel electrophoresis [26]. Electrophoresis was carried out in 0.5 X TBE buffer at 30 mA for 2-3 h (constant voltage equivalent to 3 V/cm) to allow proper resolution. Subsequently, the gel was stained by EtBr at a final concentration of 0.5 µg/ml and documented under UV light at short wavelength (254 nm) using a gel documentation system (Alphaimager). Serial dilution of uncut Lambda DNA (25-150 ng/µl) was used as a standard to quantify genomic DNA. The extracted genomic DNA was stored at -20 °C for AFLP analysis.

AFLP analysis

The AFLP procedure provided by Vos *et al*. [27] with some modifications was followed. The genomic DNA of 100 ng/µl was digested using two restriction enzymes, *Eco*RI and *Mse*I (New England Biolabs, USA), in 10x buffer A (Promega) and incubated for 1 h at 37 °C. After digestion, the restricted DNA fragments were ligated to an *Eco*RI-adapter and *Mse*I-adapter using T4 DNA ligase (New England Biolabs, USA) in order to generate a DNA template for PCR amplification. The completeness of the ligation process was detected by loading 5 µl of ligation reaction and 1 µl of 6x loading dye in 1% agarose gel electrophoresis in a 0.5x TBE buffer. Each ligation reaction was diluted 10-folded with sterilized distilled water and the aliquots were stored at -20 °C. Five microliters of the 1:10 diluted DNA template was first pre-amplified (Px2 Thermal Cycler; Thermo Electron Corporation, USA) using *Eco*RI+A and *Mse*I+C primers with 1 selective nucleotide at the 3′ end. The pre-amplification was conducted using the following cycling parameters: 30 s denaturing at 94 °C, 60 s annealing at 56 °C and 60 s extension at 72 °C.

These parameters were repeated for 20 cycles to complete the pre-amplification reaction. Then, the pre-amplified DNA was diluted to 1: 9 with sterilized distilled water and 3 µl of the pre-amplified product was used for selective amplification in a reaction tube containing 20 µl of selective amplification using *Eco*RI and *Mse*I primers with 3 selective nucleotides at the 3′ end. The selective PCR amplification reaction was performed using the following cycling parameters: 36 cycles of 30 s denaturing at 94 °C, 30 s annealing and 60 s extension at 72 °C. Annealing was initiated at a temperature of 65 °C, which was then reduced by 0.7 °C for the next 12 cycles and maintained at 56 °C for 23 subsequent cycles. The final PCR products were run on a 4.5% denaturing polyacrylamide gel electrophoresis in a 1x TBE buffer in a Sequi-Gen GT Sequencing Cell (Bio-Rad, USA). DNA fragments on the gels were stained with silver nitrate [28]. The gels were purged with distilled water and air-dried on mirror plates, and then the AFLP fragments were analysed.

Data analysis

The AFLP fragments were visually scored as 1 for present ones and 0 for the absent ones, in order to create a binary data, set as discrete variables for genetic similarity analysis. Jaccard's coefficient of similarity [29] was calculated for all pair-wise comparisons among the 8 samples of *Suaeda maritima* as follows:

$$\text{Jaccard} = N_{AB}/(N_{AB}+N_A+N_B)$$

where, N_{AB} is the number of fragments shared by two cultivars (*A* and *B*), N_A represents amplified fragments in cultivar *A*, and N_B represents fragments in cultivar *B*. The Unweighted Pair Group Method of the Arithmetic Average (UPGMA) was used to construct a dendrogram, clustering by FreeTree software [16]. To evaluate the strength of the resulting branches, bootstrap probabilities were calculated by NTSYS-pc software (version 2.02,2000) using 1,000 bootstrap resampling pieces of data.

RESULTS AND DISCUSSION

A total of 24 primer combinations were screened amongst which 12 combinations produced visible and clear bands in 8 plant samples (Table 1). The results demonstrated that different primers generated different fragment numbers and different lengths. A total of 711 amplified fragments, ranging from 50 to 500 base pairs in size, were generated from 12 primer combinations (Table 1). The bands that were produced from the 12 primer combinations ranged from 39 to 76 bands with an average of 59.25 bands per primer combinations and generated an average 87.95 percentage of polymorphic bands. The highest number of the amplified fragments was obtained from the primer pair E+AG/M+CAG (76 bands) (Fig 1), while the lowest number was obtained from the primer pair E+AAC/M+CGA (39 bands) and E+AAC/M+ CAG (39 bands).

Genetic relationships

The dendrogram was generated by Jaccard's similarity matrix and the UPGMA. (Fig 2) shows the genetic relationships among the eight population of the species *Suaeda maritima* collected from eight regions of the coastal belt of Purba Medinipur district of West Bengal in India.

According to the dendrogram, two major groups were classified. The first group (I) was composed of only the population of Sankarpur with the similarity index 0.19. The second group (II) can be divided into 3 subgroups (IIA, IIB & IIC). The first subgroup (IIA) being composed of the population of Petuaghat with a similarity index 0.67. The population of Dadanpatrabarh was clustered into a second subgroup (IIB) with a 0.71 similarity index. The last subgroup (IIC) divided into two sub group series (IID & IIE). The first series (IID) composed of the population of Bankiput, Junput and Shoula with similarity index 0.78-0.83. The second series (IIE) composed of the population of Tajpur and Digha with similarity index 0.81.

The pair-wise comparisons of the AFLP profiles were based on both shared and unique amplification bands, and were used to generate a similarity index. Among the eight populations (accessions) of the species *Suaeda maritima*, the genetic similarity ranges from 0.0.19 to 0.83 (Table 2). The population of Bankiput and Junput showed the highest genetic similarity value (0.83), whereas, the population of Sankarpur showed the lowest genetic similarity with all other populations (0.19).

Comparative studies using PCR-RFLP, RAPD and AFLP techniques have revealed that AFLP techniques are the most efficient and effective due to their high reproducibility, high quantity of information throughout multiple loci in the genome, and high resolution [15-16]. The AFLP data was used

as molecular characters for phylogenetic analyses to reveal the evolutionary relationships among the taxa [30]. The AFLP technique is commonly applied for analysis of genetic relationships and genetic diversity in *Curcuma comosa* [17], *Punica granatum* [31] and *Panax notoginseng* [32]. AFLP technology being highly reproducible due to its stringent amplification procedure [33-34], these markers were used for the first time to analyse the genetic variability and to establish phylogenetic relationships among different populations of *S. maritima* growing in different salt-enriched sites of Purba Medinipur of West Bengal in India. Prinz *et al*. [35] reported the effective nine AFLP primer combinations for the coastal and inland populations of *Suaeda maritima* of Central Europe which produced 243 scorable bands ranging in size from 32 to 412 bp and 15 to 33 bands per primer pair. But in this study, 12 different primer combinations were used for *Suaeda maritima* which produced a total of 711 clear and reproducible amplified bands ranging in size from 50-500 bp with an average of 59.25 per primer combination might be considered as more effective for these *Suaeda maritima* materials. The high percentage (87.95%) of polymorphism per primer combination indicates that there is a genetic diversity among the eight population of *S. maritima* collected from different regions of Purba Medinipur in India. Bootstrap analysis expressed that the dendrogram was created based on the genetic similarity index amongst the eight population of *S. maritima* was stable and robust. The genetic variation obtained from the populations of *Suaeda maritima* in the present study were quite higher than the coastal and inland population of *Suaeda maritima* in Germany [36]. Similar kind of diversity was also inland and coastal populations of another annual halophytic species *Salicornia ramosissima* in the Chenopodiaceae [37].

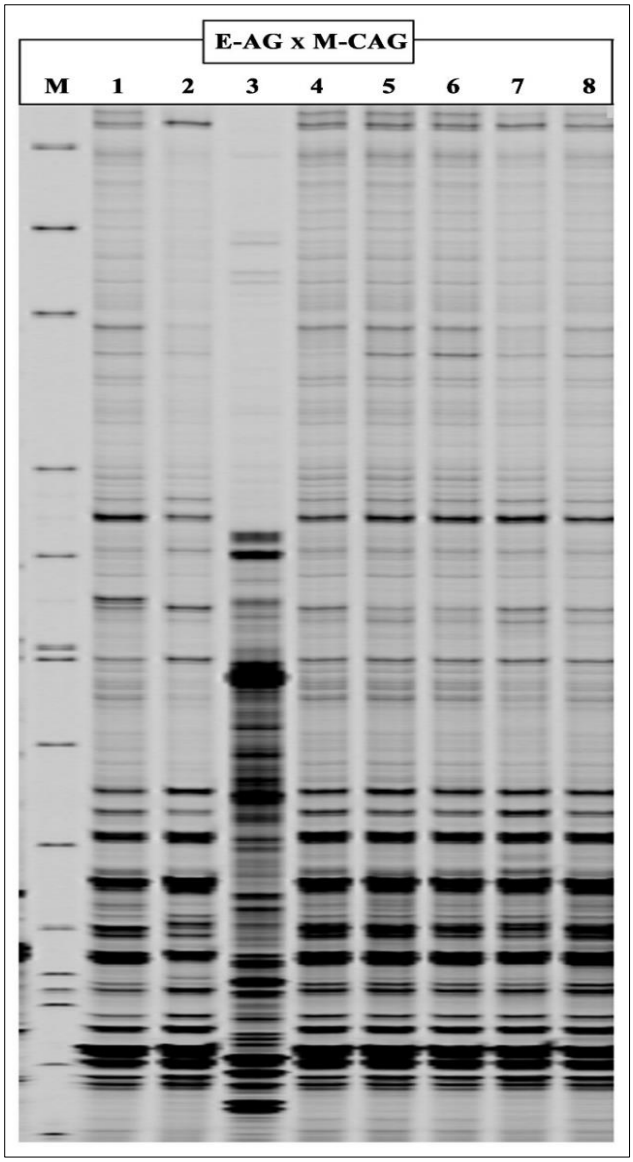

Fig 1 AFLP fingerprint of *Suaeda maritima* obtained from E+AG/M+CAG primer combination

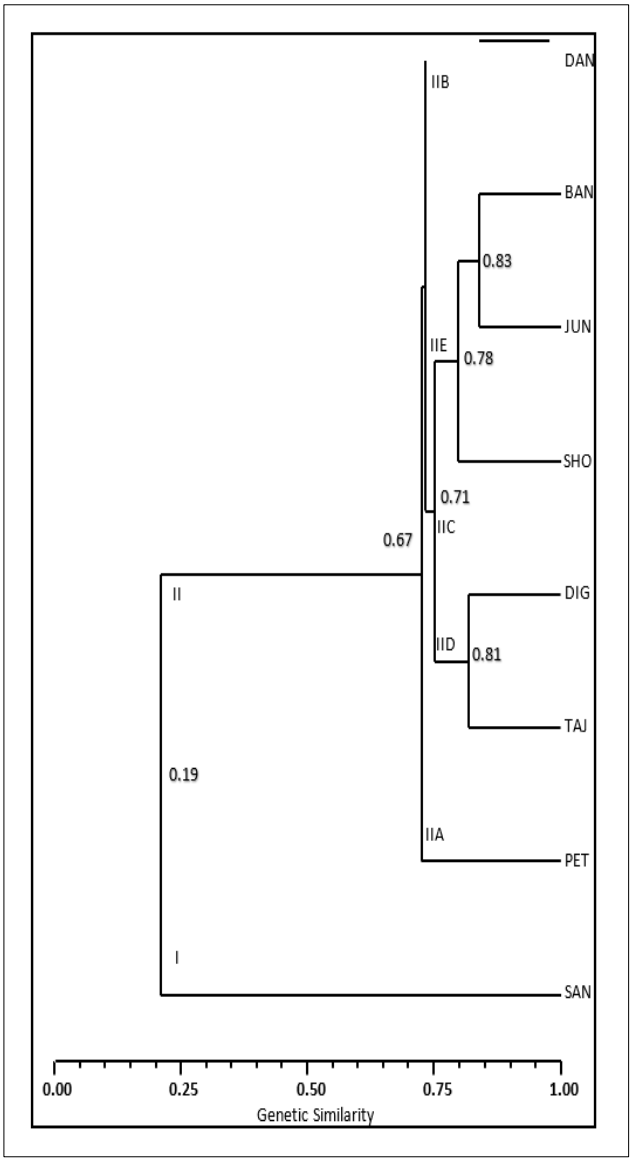

Figure 2 Cluster analysis showing the relative distances between the members of different populations of *S. maritima* with respect to AFLP performance

Clustering of *Suaeda maritima* populations, in earlier works done based on RAPD [38] and microsatellite markers [39] also produced similar nature of dendrogram as was obtained in this work with 8 populations of *Suaeda maritima*.

The variation revealed in the present study in genetic constituents of the sea shore dwelling halophyte *Suaeda maritima* depending on close or remote placement of populations gives an insight to the fact that even though they are growing almost contiguously near sea coast, with the increase of distance between the populations the difference in Amplified fragment length polymorphism (AFLP) performance also intensifies. Such dynamism might be occurring due to different extent of pressure for adaptation of the plant species at different sites of their growing.

Table 1 The list of 12 primer combinations and the number of AFLP bands, size ranges and percentages of polymorphic bands along with average

Primer combination	Number of AFLP band	Size range (bps)	Percentage [%] of polymorphic band
E + AG/M + CAG	76	50-500	88.16
E + ACA/M + CTA	64	50-500	82.81
E + AAC/M + CGA	39	50-500	97.43
E + AAC/M + CAG	39	50-500	94.87
E + AG/M + CTA	64	50-500	84.37
E + AG/M + CGA	60	50-500	83.33
E + ACA/M + CGA	75	50-500	89.33
E + ACA /M + CAG	60	50-500	86.66
E + AAC/M + CTA	52	50-500	90.38
E + AT/M + CAG	68	50-500	89.70
E + AT/M + CGA	55	50-500	83.63
E + AT/M + CTA	59	50-500	84.74
Total	711	50-500	1055.41
Average / primer combination	59.25	50-500	87.95

Table 2 Similarity matrix based on Jaccard's similarity coefficient of AFLP data of *Suaeda maritima*

	DAN	PET	SAN	BAN	JUN	DIG	TAJ	SHO
DAN	1.000							
PET	0.729	1.000						
SAN	0.199	0.203	1.000					
BAN	0.751	0.772	0.211	1.000				
JUN	0.738	0.756	0.200	0.838	1.000			
DIG	0.722	0.677	0.214	0.800	0.764	1.000		
TAJ	0.712	0.691	0.216	0.717	0.734	0.819	1.000	
SHO	0.740	0.731	0.221	0.803	0.788	0.766	0.741	1.000

DAN - Population of Dadanpatrabarh, PET - Population of Petuaghat, SAN - Population of Sankarpur, BAN - Population of Bankiput, JUN - Population of Junput, DIG - Population of Digha, TAJ - Population of Tajpur, SHO - Population of Shoula

CONCLUSION

The relationship based on AFLP performance among populations of *Suaeda maritima* showed different extent of proximity and remoteness between the populations of different locations. While no convincing point is there to expect genetic difference between contiguously growing individuals in otherwise arbitrarily set populations, the difference in AFLP might be significant in expressing dynamism in some segments of genetic contents to cope up with the environment unique for the site of their growing. These relationships did not also comply with the distance between geographical locations of the populations under consideration, as the environmental versatility of sea coast can hardly corroborate with the distance along with it. However, if this variation persists, in course of time that may lead to considerable changes in this species itself.

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