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RESEARCH ARTICLE

A study on Population structure delineated with Inter Simple Sequence Repeat markers in fragmented populations of legume tree Albizia procera.

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Abstract

Albizia procera (*A. procera*) is a popular multipurpose legume tree with a wide distribution and is highly valued for its quality timber and gum yield. However, the species has been overexploited and could be traced as fragmented populations. To estimate the partitioning of genetic variation, ISSR markers were used to analyze 102 individuals representing six natural populations of the species distribution range. Estimates of genetic differentiation between-population were strongly correlated with geographical distance ($r = 0.89$, $p < 0.001$), which, along with a UPGMA phylogenetic analysis, strongly suggested high levels of genetic isolation by distance. Taken together, these results suggest that fragmentation has led to an increase in population differentiation between fragments of *A. procera*. These formations will be of great value in the development of conservation plans for species exhibiting high levels of genetic differentiation due to fragmentation, such as indication of conservation unit size, which populations should be chosen as priority in conservation plans and which samples should be introduced in areas with less

Introduction:-

Excessive and accelerating levels of overexploitation have led to the fragmentation of many natural populations. Forest fragmentation is a disruptive process that led to loss of habitat and biodiversity. There will be an increase in inbreeding and drift due to decrease gene flow between fragments of a once-continuous population. This may account for a decrease in the level of genetic variation both at local scale and over entire species range. Population genetic theory predicts that decrease in the genetic diversity limits a species ability to keep pace with the changing selection pressure and ultimately leaves the population less able to adapt to changes in its environment, increasing the risk of extinction (Keller and Waller 2002)**.** Thus, understanding of genetic variation among populations and species genetic structure is useful and crucial for optimizing sampling strategies, administration and conservation of genetic resources. Indian tall siris, *A. procera* (Roxb.) Benth. belongs to sub-family Mimosacae of family Leguminosae. It is native to moist deciduous, semi-evergreen hill forests, swamp forests and lowland savanna woodlands in Asia and northern Australia (Parrotta 1988b). *A. procera* is a fast-growing tree. Light- to chocolate-brown with light and dark bands wood is durable, strong and resistant to termites and is used to produce wheels, carts, boats, furniture, flooring, posts, agriculture implements, boxes and carvings. The bark yields tannins and a reddish gum and the species is considered a promising source of pulp for high-quality paper (Parrotta 1987). The leaves were used to treat ulcers and have insecticidal properties (Parrotta 1987). *A. procera* forms symbiotic association with Rhizobium bacteria enabling it to fix nitrogen and thrive on infertile soils and serves as an important reforestation and agroforestry species. Though this species has a high economic importance, the diversity was not studied. Traditionally, provenance and progeny tests coupled with biometrical analysis of phenotypic traits have been the standard methods for describing and quantifying genetic variation in forest tree species (National Research Council 1991). However, these approaches are slow, lengthy and expensive. Recent development of

molecular markers has complemented and drastically reduced the time and cost. For multipurpose tropical forest tree, obtaining quick, accurate estimates of the distribution of genetic variation in a cost effective manner is particularly important. So, ISSR were chosen as the marker system because there is no need for previous knowledge of the genome, it produces a large number of reliable and reproducible markers and requires only micrograms of DNA (Zietkiwicz et al. 1994). Despite the importance there is no conservation management plan for the existing populations. As an initial step into developing such a plan, we carried out an initial study to record the distribution of diversity within and relationships among six populations of *A.procera* from South east India using ISSR markers. The results would be helpful to identify best individuals for incorporation in reforestation and enrichment programs and also in the formation of germplasm banks.

Materials and Methods:-

Population studied:-

The study sites included six natural populations from the Northern and Eastern part of Indian sub-continent (Fig.1). The locations of the population and their eco-geographical parameters are shown in Table-1. The *A. procera* trees in the study site were mapped and a total of 102 of these were sampled. For each population the number of individuals varied from 12 to 25 for ISSR analysis.

Molecular analysis:-

Two gram of leaves was pooled from each individual tree and total DNA was isolated and quantified following the protocol given by Aparajita and Rout (2009). Twenty-two synthesized ISSR primers were evaluated to test their degree of polymorphism. Polymerase chain reactions (PCR) with single primer was carried out in a final volume of 25 µl containing 20 ng template DNA, 100 µM of each deoxyribonucleotide triphosphate, 20 ng of I-SSR primer 1.5 mM $MgCl_2$, 1x Taq buffer (10 mM Tris-HCl [pH-9.0], 50 mM KCl, 0.01% gelatin), and 0.5U Taq DNA polymerase (M/S Bangalore Genei, Bangalore, India). Amplification was performed in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) programmed for a preliminary 2 min denaturation step at 94 ^{0}C, followed by 40 cycles of denaturation at 94 ^{0}C for 20 sec., annealing at required temperature for 30 sec depending on the sequence of ISSR primer and extension at 72 ^{0}C for 1 min, finally at 72 ^{0}C for 10 min amplification. Amplification products were separated by electrophoresis alongside a molecular weight marker (1.0 Kb plus ladder, M/S Bangalore Genei, Bangalore, India) using 2 % agarose prestained with ethidium bromide and 1X TAE (Tris Acetate EDTA). The amplified products were analysed through Gel Doc System (Gel Doc. 2000, BioRad, California, USA). Reproducibility and repeatability of the amplification products were tested thrice for each of the primers.

Statistical analysis:-

Although the visualizations varied in size, the results from each ISSR product were assumed to represent a single locus. Only data from intensely labeled, unambiguously clear bands were used for statistical analysis. Data was coded on a binary basis (1-presence of band; 0-absence of band) and entered in the form of single individual genotypes, following the specification of the population genetic software package POPGENE Ver.1.31 (Yeh et al., 1999). Within-population diversity values including the mean number of alleles per locus (A) and the percentage of polymorphic loci (P) were calculated using Nei's unbiased diversity statistic (Nei 1987) averaging over ISSR products. The level of gene flow (Nm) was measured using Nei's (1973) gene diversity statistics. The Shannon information measure (Io) was calculated at two levels: the average diversity within populations (Ipop), and the total diversity within species (Isp). The proportion of diversity between populations D = (Isp- Ipop)/Isp was estimated. A UPGMA (Sneath and Sokal, 1973) dendrogram showing the relationships between populations based on Nei's genetic distance (Nei 1978) was constructed. The genetic distance matrix was also used to perform a hierarchical analysis of molecular variance (AMOVA; Excoffier et al., 1992) essentially as described by Huff et al., (1993) using the Arlequin software package (V2; Schneider et al., 2000). The correlation between genetic and geographical distances was analysed using the Mantel test option implemented in the Arlequin package.

Results:-

ISSR marker polymorphism:-

In the 102 individuals, the sixteen primer combinations used generated 236 unambiguous, scorable and highly polymorphic fragments. Out of a total of these 236 bands scored 216 (91.5%) were polymorphic at the species level, either between or within populations, across our entire sample. The ISSR primer IG-15[$(GA)_8T$] showed the highest number of product (21 amplicons) and least was obtained in IG-18[$(CT)_{8A}$] (05 amplicons) with an average of 14.75 bands per primer. The primers differed in their ability to detect polymorphism. The percentage of polymorphic bands amplified per primer overall population varied from 79.1% (IG-23) to 100% (IG-13) and for individual population it ranges from 31.66% (PCB population) to 45.05% (PMP population).

Phylogenetic relationships among populations:-

The genetic distance matrix (Nei 1978) was used to establish the level of genetic divergence between the populations (Table 4). Estimates of genetic distance using ISSR data ranged from 0.09 for the most closely related populations (PBC and PSC) to 0.4635 in the most divergent populations (PDH and PBC). A UPGMA dendogram based on the genetic distance was constructed to graphically visualize the genetic relationships among the populations (Fig.2). The six populations were separated into three major groups: the first group consisted of two populations PMP and PMH, the second group consisted of three populations PSC, PBC and PCB, while population PDH was clustered alone as a separate group. The matrix of the genetic distances correlate significantly with the corresponding matrix of geographical distances (Mantel test, correlation coefficient of $r=0.89$ and a significance of $p<0.001$).

Genetic variation within and among population:-

Estimates of Nei's genetic diversity (Nei 1987) for all loci in individual populations showed most diversity in malyagiri plain ($H = 0.22$) and least diversity in Deheradun ($H = 0.12$). The mean diversity for all the population was 0.14 and the pooled species level value was estimated to be 0.36. The mean percentage of polymorphic loci for all population was 34.07% and the pooled species level value was 85.5%. Data on the allelic frequency, percentage of polymorphic products along with the estimates of Nei's genetic diversity for all loci in individual populations are given in Table 2. The coefficient of genetic differentiation between populations was 0.5136 as estimated by partitioning of the total gene diversity (Gst). The level of gene flow (based on Gst) estimated was very low ($Nm = 0.36$); the Shannon's diversity index (D) was 49.82%. The AMOVA analysis provided corroborating evidence for the genetic structure obtained from Nei's genetic diversity statistics and Shannon's diversity estimation. The total variance analyzed was 34.16% between populations and 65.84% between individuals within a population ($P<0.001$) (Table - 3).

Discussion:-

The primary aim of this study was to test the effect of forest fragmentation on the levels and patterns of genetic diversity in the tropical legume tree Albizia procera. The high level of observed polymorphic loci amplified in *A. procera* (91.5%) corroborate with figures reported for other tropical trees like Euterpe edulis (Cardoso et al. 2000); Caesalpinia echinata Lam. using AFLP (Cardoso et al. 2004) and in Albizia lebbeck using ISSR (Aparajita and Rout, 2009). This suggests a high level of genetic variation in Albizia procera. Despite this, the proportions of polymorphic loci for some of the populations were quite low (between 33.6% in Deheradun to 50.5% in Malyagiri plain). Nevertheless, at the species level the results are consistent with data from other tree species, in which high genetic variation has been related to life history and ecological characteristics such as a wide geographical range, primarily out crossing and animal-seed dispersal mechanisms (Hamrick & Loveless 1989). In some tree species, a lower percentage of polymorphic loci were also observed as in Theobroma cacao using RAPDs (Russell et al. 1993), in Moringa oleifera using AFLPs (Muluvi et al. 1999) and in Glyptostrobus pensilis (Li and Xia 2004) using ISSR markers. Such results are in contrast to exceptions for woody, long lived, and predominantly outcrossed species, which are expected to maintain most variation within populations (Hamrick 1989). Despite all this fact, our result also showed high levels of population structure and taken together the results from UPGMA analysis, the mantel test and the AMOVA all strongly suggest genetic isolation by distance. The correspondence between genetic relationships inferred from the phylogram and geographical relationships is confirmed by the Mantel test and suggests that restricted gene flow between populations is the major factor responsible for the observed differentiation. The highest levels of within-population genetic diversity were found within the Malyagiri plain (PMP) and Chandaka Bhubaneswar (PCB) population. Distribution range and population size have been identified as major correlates of within population genetic variation in tropical tree species, with restricted populations

showing significantly less variation than those with broader distribution (Travis et al. 1996). On the other hand, Deheradun (PDH) population which is represented by a large population, showed lower values of genetic diversity. This result indicates that levels of genetic diversity do not depend solely on sample size but on several factors, including founder effects and genetic bottlenecks (Suyama 2000). Despite these results, however, it is difficult to determine whether these patterns of genetic diversity are the result of recent, human-mediated fragmentation or longer-term, biogeographical factors.

The ultimate goal of conservation is to ensure the continuous survival of populations and to maintain their evolutionary potential (Godt and Hamrick 1995). The present analysis, based on populations representing the main regions where the species is found, can provide a foundation for the proposal of rational conservation management plans for *A. procera*, as well as for species which display levels of fragmentation and genetic differentiation. We are aware that it is very hard to select all populations as priority areas for conservation units, as the Bhubaneswar Chandaka population (PCB) and population from coastal regions (PBC and PSC) suffers an intense pressure due to urbanization. The most divergent populations harbour genetic variation that could be found in any of the other populations and this should be taken into account when managing the species at the population level. The Malyagiri plain (PMP) population should be selected because it presents the highest genetic variation and the most preserved population. Genetic differentiation provides a template for adaptation and evolution of populations and species; therefore the maintenance of high genetic diversity in *A. procera* is one of the most important issues for sustainable *A. procera* forest in the future. In future, we plan to carry out additional studies to explain the contribution of ecological and biological factors, as well as the impact of habitat fragmentation of Indian tropical forest, on the observed genetic structure. In this respect, advanced molecular markers like AFLP and microsatellites may provide the most useful tool for better understanding the mating system and gene flow in tropical forest trees.

Legends to the Figures

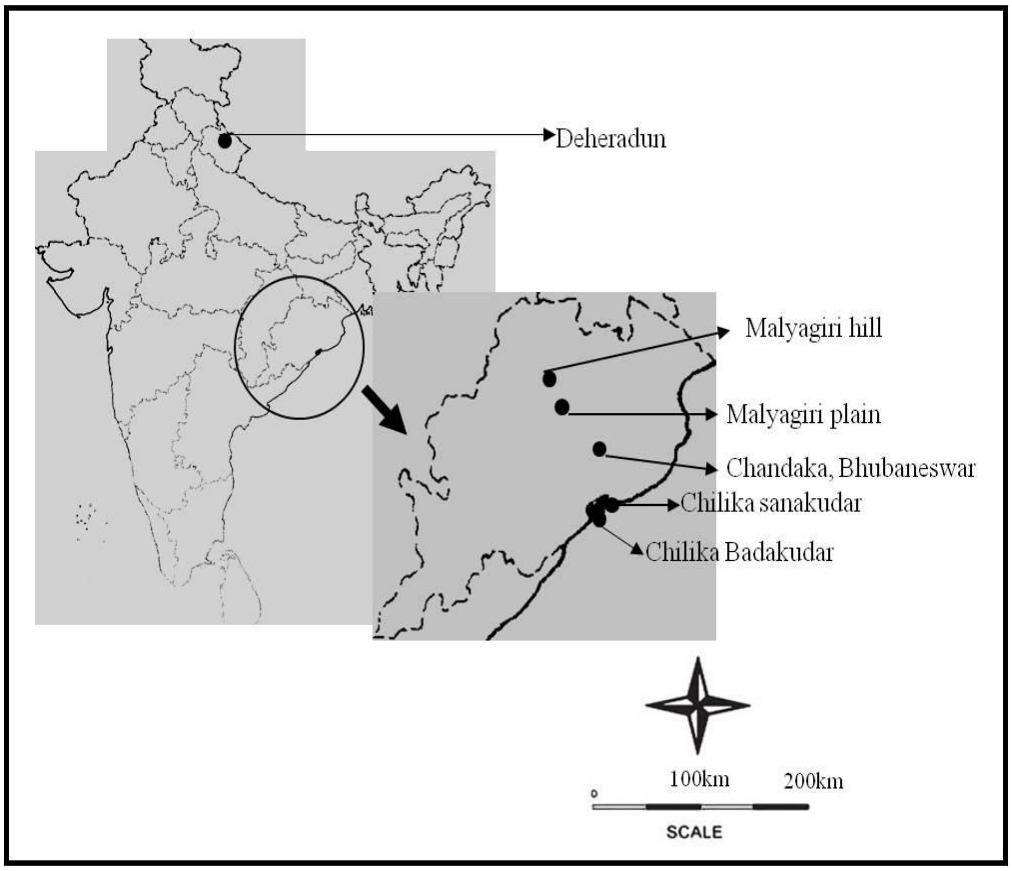

Figure 1. Location of A.procera populations sampled in this study

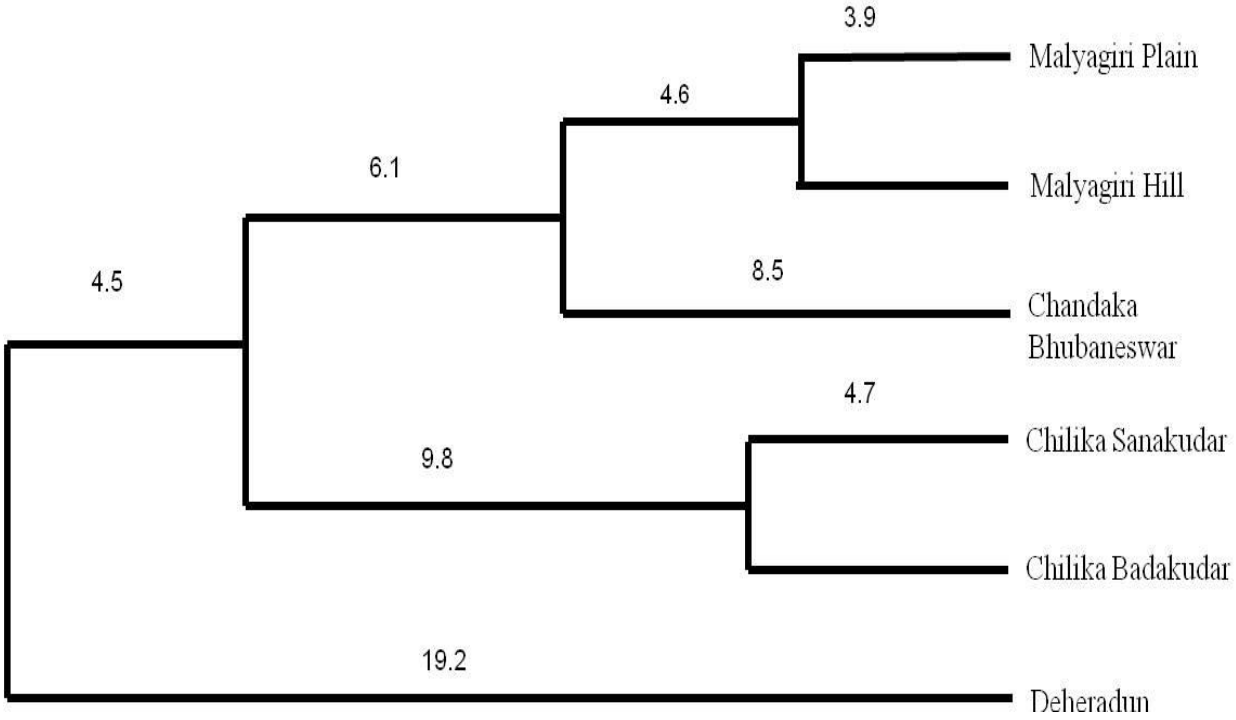

Figure 2. UPGMA phylogenetic tree showing relationships between populations based on Nei's genetic distance.

Table 1. Eco-geographic data of different locations from which *A. procera* collected:-

Location	Status of stand	N	GPS reading	Altitude (mt.)	Climatic parameter	
					Tm (oC)	Rn (cm.)
Malyagiri Plan (PMP)	Natural forest	19	$21^0$07', $85^0$29'	1060	25.0-29.5	200-400
Malyagiri Hill (PMH)	Natural forest	16	$21^0$32', $85^0$29'	1430	25.5-32.5	40-100
Sanakudar Chilika (PSC)	Natural forest	14	$19^0$48', $85^0$49'	60	25.0-30.5	100-200
BadakudarChilika (PBC)	Natural forest	12	$19^0$48', $84^0$12'	50	25.0-30.5	100-200
Chandaka Bhubaneswar (PCB)	Natural forest	16	$20^0$08', $85^0$58'	220	25.0-30.5	100-200
Deheradun (PDH)	Protected forest	25	$30^0$18', $76^0$03'	3070	22.0-29.5	80-120

Table 2. Average diversity values detected for the Populations of *A. procera* with ISSR markers. (N-Sample size, %-percentage of polymorphic loci, Ae- effective allele frequency, Ho- nei's genetic diversity, Io- Shannon's indices):-

Populations	N	P %	Ae	Ho	Io
Malyagiri Plan (PMP)	19	45.05	1.36 (0.40)	0.22 (0.20)	0.32 (0.29)
Malyagiri Hill (PMH)	16	38.26	1.33 (0.42)	0.17 (0.22)	0.28 (0.29)
Sanakudar Chilika (PSC)	14	36.87	1.36 (0.43)	0.19 (0.21)	0.22 (0.30)
Badakudar Chilika (PBC)	12	31.66	1.32 (0.41)	0.17 (0.20)	0.21 (0.30)
Chandaka Bhubaneswar (PCB)	16	40.07	1.42 (0.38)	0.20 (0.23)	0.29 (0.29)
Deheradun (PDH)	25	34.07	1.22 (0.41)	0.12 (0.19)	0.26 (0.31)
Population Average	17		1.27 (0.40)	0.14 (0.21)	0.25 (0.30)
Species Average	102	82.35	1.65(0.30)	0.37 (0.14)	0.19 (0.28)

Table 3. Analysis of Molecular Variance (AMOVA) for 102 individuals in six population of *A. procera* using ISSR markers:-

Source of variation	Degree of freedom	% total variance	p-value
Among population	5	34.16	<0.001
Within population	96	65.84	<0.001

Table 4. Genetic distance between six natural populations of *A. procera* based on ISSR data. (Population codes are as in Table 1):-

Population	PMP	PMH	PSC	PBC	PCB	PDH
PMP	-					
PMH	0.078	-				
PSC	0.282	0.333	-			
PBC	0.293	0.385	0.096	-		
PCB	0.182	0.160	0.233	0.234	-	
PDH	0.369	0.348	0.391	0.463	0.351	-

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