Bangladesh J. Bot. **50**(2): 405-412, 2021 (June)

GENETIC DIVERSITY WITHIN AND AMONG POPULATIONS OF *SHOREA ROBUSTA* ROXB. EX GAERTN. IN BANGLADESH AND ITS IMPLICATIONS FOR CONSERVATION

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Keywords: Genetic diversity, ISSR marker, Population, *Shorea robusta*

Abstract

Fragmentation and reduction of natural population size render threats to the conservation of forest resources through depletion of genetic diversity. Hence, information on genetic structure of Sal (*Shorea robusta* Roxb. *ex* Gaertn.) populations is relevant for proper management and conservation of the tropical deciduous forests. The present study focused on assessing the genetic diversity of the populations of Sal which was the dominant tree species of the deciduous forests of Bangladesh. Plant leaf samples were collected from the three populations of Sal distributed in the three geographical regions including Madhupur tract in the districts Tangail and Gazipur and that of the districts of Cumilla and Dinajpur. DNA band profiles were generated using eight ISSR primers for a total of 13 samples taken from the three populations. Statistical analysis was done using PopGen 32 and GenAlEx 6.5 softwares. Principal coordinate analysis done on the DNA band profiles revealed that Sal populations of Madhupur tract and Cumilla positioned nearby while Dinajpur showed maximum genetic distance with that of Cumilla. Mantel test showed significant (p=0.05) correlation between genetic and geographic distances indicating "Isolation by Distance". Data of the present study indicated higher genetic polymorphism (68.87%) in the Sal population of Madhupur tract compared to other two populations. Small population size of Sal of Dinajpur forest might be related with its low genetic diversity. Data of the present study suggest immediate attention for the conservation of Sal forests in Bangladesh before further genetic erosion occurs.

Introduction

Sal (*Shorea robusta* Roxb. *ex* Gaertn.) belonging to Dipterocarpaceae is the dominant tree species in the deciduous forests that are distributed in the south Asian countries including Bangladesh, Bhutan, Nepal and India covering an area of about 12 million ha (Surabhi *et al.* 2017). This tree species is native to the Indian subcontinent ranging in the south of the Himalaya, from Myanmar in the east to Bangladesh, Nepal and India in the west. In Bangladesh, Sal forests currently cover an area of about 1,20,000 ha distributed sporadically in different geographical locations (Alam *et al.* 2008). Over the decades, these forests have been fragmented rapidly due to anthropogenic activities causing land-use changes and indiscriminant logging (Hossain *et al.* 2013). Genetic erosion in natural populations resulting from fragmentation and isolation may reduce reproduction and survival potentials rendering population viability at risk since genetic variation help plants respond to environmental pressure, evolve, and survive in the long term (Wei *et al*. 2005). However, the consequence of fragmentation and isolation of Sal population at its genetic level has not yet been studied in Bangladesh although such information is relevant for the proper management and conservation of the forest species.

Inter Simple Sequence Repeat (ISSR) markers also known as microsatellites are reported to be dispersed throughout genomes anchored either at 5′ or 3′ end with one or few specific nucleotides and amplify the sequences between two microsatellite loci (Hu *et al.* 2010). Because

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DOI: https://doi.org/10.3329/bjb.v50i2.54098

of the higher annealing temperature and longer sequence of ISSR primers, they can yield more reliable and reproducible bands than rapid amplified polymorphic DNA (Goulão and Oliveira 2001). A set of ISSR primers were used as they are neutral to environmental factors and have more polymorphic information content (Blair *et al.* 1999). Considering the immense ecological and economic importance of *S. robusta*, the present study aimed to assess the genetic variation between and among populations of Sal, to ascertain the impact of population size on genetic diversity and also to examine the relationship between genetic variation and geographic distance.

Materials and Methods

For the collection of leaf samples, natural populations of Sal were selected from the forests in the Madhupur tract under the districts of Tangail and Gazipur and those in the districts of Cumilla and Dinajpur (Fig. 1). Geographic location of each sample site of the study area is shown in Table 1. A total of 13 locations, 7 from Madhupur tract (Gazipur and Tangail) and each three from Cumilla and Dinajpur forests were selected in the present study to collect samples. The three forests varied in average precipitation, humidity and temperature; the precipitation rate of Madhupur tract, Cumilla and Dinajpur were 1872, 2295 and 1728 mm, respectively, likewise the humidity was 70, 75 and 69%. The forestlands also differed in soil type: Madhupur tract is clayey, Cumilla is sandy loam and Dinajpur is rich in lime (Banglapedia 2015). Fully expanded youngest leaf samples were collected from the plants for the extraction of DNA. During leaf sample collection, minimum 50 m distance was maintained among sampling locations within each of the three populations. After completion of sampling, leaves were labeled separately and kept at -20ºC until DNA extraction was carried out.

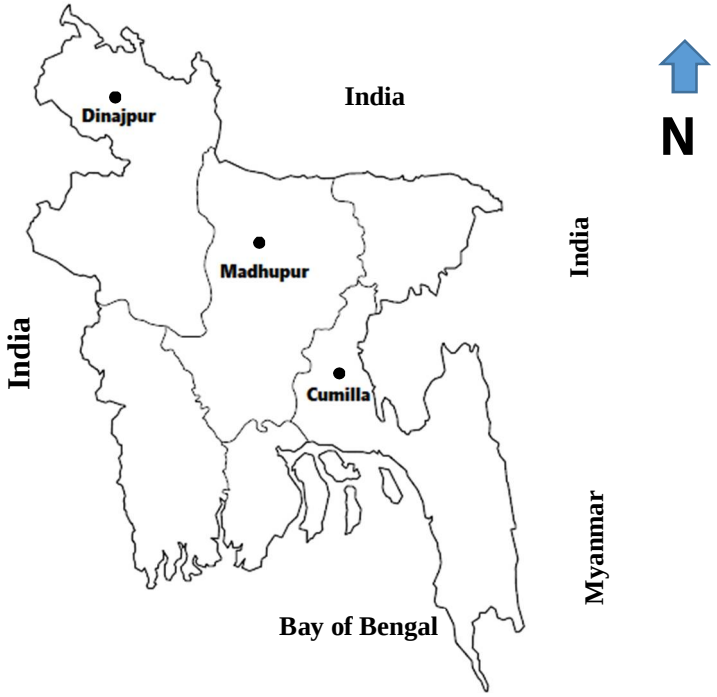

Fig. 1. Map showing the distribution of populations (round fill) of Sal (*Shorea robusta*) selected for the study.

Table 1. Geographic information of the locations from where plant leaf samples were collected for the extraction of DNA.

Population	Location	Latitude	Longitude
Madhupur Tract	Gazipur	24º4'22.48"	90º23'30.01"
	Madhupur National Park	24º41'24.47"	90º8'1.5"
	"	24º41'25.8"	90º7'59.45"
	"	24º41'25.84"	90º7'56.28"
	Jalchatra	24º38'35.2"	90º4'55.67"
	"	24º38'34.04"	90º4'55.6"
	"	24º38'32.5"	90º4'55.09"
Cumilla	Shalban Bihar	23º25'17.9"	91º8'12.26"
	"	23º25'19.06"	91º8'13.02"
	"	23º25'18.44"	91º8'10.36"
Dinajpur	Singra Forest	25º53'23.41"	88º33'37.91"
	"	25º54'43.09"	88º33'58.21"
	"	25º53'15.61"	88º33'48.92"

For the extraction of DNA, 500 mg of fresh leaf material was used. At first, leaves were washed and sterilized with distilled water and 70% alcohol, respectively and then rinsed with distilled water to remove alcohol. Leaves were ground using liquid nitrogen. DNA was extracted following a modified CTAB method (Milligan 1992). Eight ISSR primers were selected based on their ability to produce distinct and maximum polymorphic amplified products (Surabhi *et al.* 2017). DNA sequence, annealing temperature and number of alleles of each primer are presented in Table 2.

Table 2. List of ISSR primers along with the number of bands produced per primer and annealing temperature detected in *Shorea robusta* (Surabhi *et al.* 2017) used in the present study.

Sl. No.	Primer ID	Primer Sequence (5'-3')	Amplified band	Temp. (°C)
1	$(AC)_8C$	ACACACACACACACACC	21	54.8
2	$(GA)_8YT$	GAGAGAGAGAGAGAGAYT	15	52.1
3	$(GA)_8YG$	GAGAGAGAGAGAGAGAYG	11	52.9
4	$(AG)_8YT$	AGAGAGAGAGAGAGAGYT	15	50
5	AGGG	AGGGCTGGAGGAGGGC	16	49.8
6	$(AG)_8C$	AGAGAGAGAGAGAGAGC	10	52.8
7	$(CT)_8G$	CTCTCTCTCTCTCTCTG	12	46.8
8	$ACG(GT)_7$	ACGGTGTGTGTGTGTGT	6	54.5
		Total =	106	

The PCR reaction mixture consisted of 50 ng of template DNA, 12.5 µl of PCR master mix (1 mM of each dNTP, 1.5 mM $MgCl_2$, 1-unit Taq polymerase), 1µl primer and 8.5 µl of PCR-grade sterile de-ionized distilled water. The PCR amplification was done using a thermal cycler (Applied

Biosystems 2720 Thermal cycler, USA) as following 40 cycle of initial denaturing step of 2 minutes at 94ºC, denaturation step at 94ºC for 30 sec, annealing step of 1 min and extension and final extension at 72ºC for 2 and 7 min, accordingly. After the completion of PCR, gel electrophoresis was performed by separating the DNA in 1.5 % (w/v) agarose gel stained with ethidium bromide. Solidified gel was placed into gel-running kit 1x TAE buffer. Molecular marker of 1.0 kb Plus DNA ladder was electrophoresed alongside the reaction samples to compare and score band size. DNA bands were observed on UV-transilluminator and photographed by a gel documentation system (Cleaver Scientific's MultiSUBTM, UK).

Each experiment was run twice in case of every primer to avoid any dubious result. Clear and reproducible bands were scored as binary data on the basis of their presence (1) and absence (0). Observed number of alleles (No), effective number of alleles (Ne), percentage of polymorphism (%), allelic richness, number of polymorphic loci, Nei's gene diversity (Nei 1973) and Shannon's Information index (Lewontin 1972) were calculated using Popgene version 1.32 software. Analysis of molecular variance (AMOVA) was used to estimate the partitioning of genetic variance among and within populations using GenAlEx6.5 (Peakall and Smouse 2006). Principal Coordinate (PCO) analysis was also performed to find out the association among individuals from different geographic populations. A measure of Isolation by Distance (IBD) was obtained by plotting genetic distance values against log transformed geographic distance values and by performing a mantel test using the software GenAlEx 6.5 (Peakall and Smouse 2006). Nei's unbiased measures of genetic identity and genetic distance (Nei 1973) were performed by PopGen32 software.

Results and Discussion

The eight ISSR primers produced a total of 106 bands ranging from 6 to 21 alleles for different primers (Table 3). Among all the 8 primers, $(AC)_8C$ produced the highest number of bands which made it the most informative. On the other hand, primer $ACT(GT)_7$ yielded the lowest number of bands for the samples ranging from 900 to 350bp. The DNA band profile of primer $(AC)_8C$ in all three populations is shown in Fig. 2.

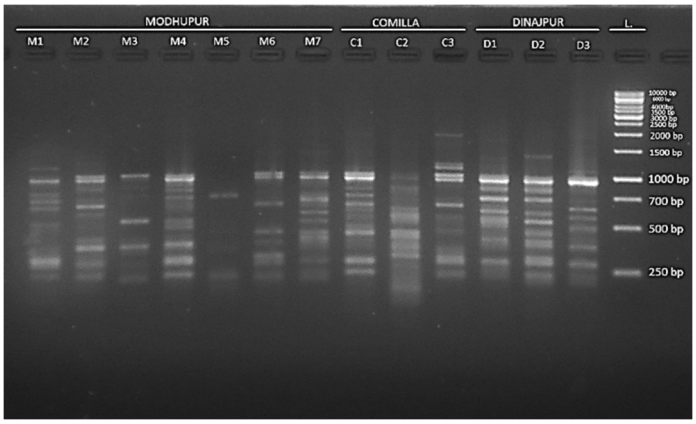

Fig. 2. Banding pattern of DNA in 13 samples of *Shorea robusta* using primer 5'-ACACACA CACACACACC-3' and L is the ladder of 1kb sized marker.

The highest polymorphism (68.87%) was found in Madhupur and the lowest (27.36%) was found in Dinajpur population (Table 3). The effective number of alleles (Ne) ranged from 1.6887

in Madhupur to 1.2736 in Dinajpur. Gene diversity (h) showed the highest value in Madhupur and the lowest in Dinajpur with a mean value 0.1886. Furthermore, Shannon's information index (I) ranged from 0.404 to 0.1741 with an average value of 0.2727. Highest allelic richness (Ar) was observed in Madhupur (81) and lowest in Dinajpur (64) populations.

Table 3. Summary of genetic variability within populations of *Shorea robusta* of Bangladesh by analyzing ISSR markers.

Population	Ar	Na	Ne	H	I	He	Poly-morphic loci	Poly-morphism (%)
Madhupur	81	1.6887	1.4894	0.2765	0.404	0.244	73	68.87%
		±0.4652	±0.3909	±0.2033	±0.2873	±0.019		
Cumilla	72	1.3774	1.3019	0.1677	0.2402	0.159	40	37.74%
		±0.487	±0.3896	±0.2165	±0.31	±0.021		
Dinajpur	64	1.2736	1.2189	0.1216	0.1741	0.121	29	27.36%
		±0.4479	±0.3583	±0.1991	±0.2851	±0.02		
Mean		1.4465	1.3367	0.1886	0.2727	0.175		

Ar: Allelic richness, Na: Observed number of allele, Ne: Effective number of allele, h: Nei's gene diversity, He: Expected heterozygosity.

AMOVA statistics showed a significant genetic variation ($p< 0.001$) in both within (69%) and among populations (31%) (Table 4). However, when compared between Madhupur and Cumilla forests, genetic variation was 75% within population and that was 25% between populations (Table 5). The highest amount of Nei's unbiased genetic distance was found between the populations of Cumilla and Dinajpur (0.4149) forests, followed by those of Madhupur and Dinajpur (0.3196). On the contrary, the lowest genetic distance (0.269) was found between Madhupur and Cumilla forests indicating that the maximum genetic similarity (0.7642) was between these two forests **(**Table 6).

Table 4. AMOVA in three populations of *Shorea robusta* of Bangladesh.

Source	Df	SS	MSD	Est. Var.	TV (%)	P (rand > = data)
Among Populatios	2	82.857	41.429	6.759	31%	0.0001
Within Populations	10	149.143	14.914	14.914	69%	0.0001
Total	12	232.000		21.673	100%	

PhiPT value = 0.312 (p <0 .001). df: Degrees of freedom; SSD: Sum of squared deviation; MSD: Mean sum of squared deviation; Est. var.: Estimated variance; TV %: Total variance percentage; P value: probability value.

Principal Coordinate (PCO) analysis revealed that all the individuals of their respective population formed three clear clusters. Among the three clusters, the clusters of Madhupur and Cumilla displayed the nearby positions whereas the cluster of Dinajpur was at distance (Fig. 3). The first three axes (coordinates) revealed 31.43, 25.02 and 13.29% of variation, respectively.

Table 5. AMOVA to calculate PhiPT value within and among Sal population of Madhupur and Cumilla forests in Bangladesh.

Source	Df	SS	MS	Est.Var.	TV(%)
Among Populations	1	38.690	38.690	5.349	25%
Within Populations	8	129.810	16.226	16.226	75%
Total	9	168.500		21.575	100%

PhiPT value = 0.248 ($p<0.001$) df: Degrees of freedom; SS: Sum of square; MS: Mean sum of square; Est. var.: Estimated variance; TV %: Total variance percentage.

Table 6. Nei's pair wise genetic identity (above diagonal) and genetic distance (below diagonal) among the three populations of *Shorea robusta* of Bangladesh by analyzing ISSR markers using PopGen32 software.

Population	Madhupur	Cumilla	Dinajpur
Madhupur	***	0.7642	0.7264
Cumilla	0.2690	***	0.6604
Dinajpur	0.3196	0.4149	***

r = 0.19; P = 0.05. Correlation coefficients (r) calculated by using the Mantel test is presented in the last line along with the p value.

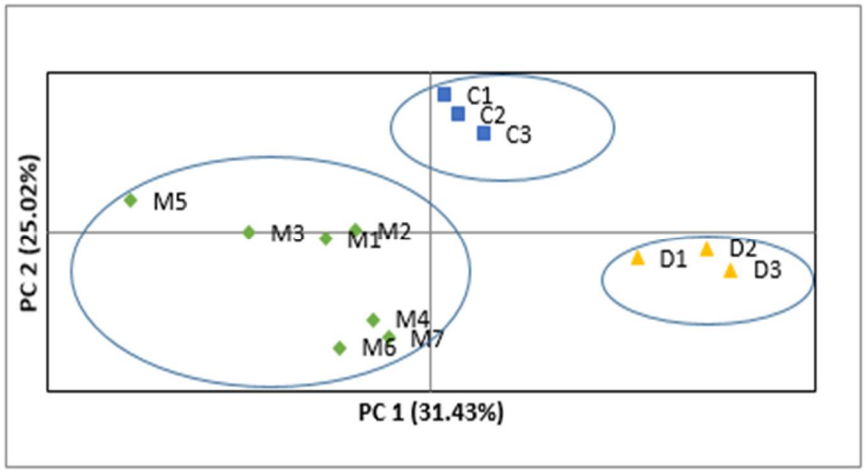

Fig. 3. Principal coordinate analysis performed using ISSR markers illustrated the samples of Madhupur (M1-M7), Cumilla (C1-C3) and Dinajpur (D1-D3) in different bi-plots.

The AMOVA statistics revealed 69% variation within population and the rest 31% of that among populations indicating a higher genetic diversity present within the populations than that present among the three populations of Sal studied. The low amount of variance among populations indicated lower level of genetic differentiation, that is, the phenomenon of speciation is not yet visible in the Sal populations taken under the present study. High level of genetic variation within population is a common situation, because out-crossing and vegetatively

propagated perennial species are generally highly heterozygous (Zhang *et al.* 2015). Due to this out-crossing nature and large population size, the percentage of polymorphism (68.87%) might be higher in Madhupur than the other two populations. Genetic diversity is also a reflection of fitness and the acquisition of adaptation. Therefore, based on this data it is quite clear that, Sal plants in the Madhupur tract possess the ability to cope up with unpredictable conditions for longer period.

On the contrary, the lowest amount of polymorphism found in the Sal forest of Dinajpur (27.36%) might be related with the small area of this forest. Total area of Singra forest in Dinajpur was about 355 ha whereas the Madhupur Sal forest was 18,439.57 ha (Khan *et al.* 2004). Some studies showed that narrowly distributed species had a lower level of genetic diversity than widely spread species (Maki and Horie 1999). Being completely isolated from other Sal populations, the Sal forest of Dinajpur was perhaps facing decreased rate of gene flow and inbreeding depression, although, further study with more data is needed to infer on this topic.

The highest amount of genetic similarity found between Madhupur and Cumilla forests was also visible in the clustering of PCO analysis where the position of these two clusters was close to each other (Fig. 3), while the cluster of Dinajpur forest was in distant position from other two clusters displaying genetic distance of this population. The reason behind the genetic similarity could be the presence of gene flow since they were geographically nearby. Overall PhiPT value of the three populations was 0.312 (Table 4) which was much higher than the PhiPT value (0.248) between Madhupur and Cumilla (Table 5) with statistical significance ($p < 0.001$). Less PhiPT value indicated lower amount of genetic difference between two geographically proximal populations. In contrast, maximum genetic distance was present between Cumilla and Dinajpur (0.4149), followed by Madhupur and Dinajpur (0.3196) forests. Mantel test revealed significant "isolation by distance". This test indicated that geographic distance had significant correlation with genetic distance (Table 6). The fragmented Sal forest of Dinajpur was much separated geographically from the other two populations and its population size was comparatively smaller. It was thought that small populations could lose large amounts of genetic variation due to genetic drift and thus had reduced probabilities of long-term viability (Lande and Barrowclough 1987). Different climatic conditions (such as relatively high temperature and low moisture) and environmental factor could make their genetic structure different from that of the other population as revealed by the position of the clusters; Dinajpur was distant from Madhupur and Cumilla populations.

As the stability and the evolutionary potential of a species depend on its genetic variation, it was important to obtain knowledge about the amount of genetic diversity to provide information for the development of strategies for conservation and sustainable utilization of a species (Cao *et al.* 2006). The current study was a first attempt in its kind to provide an insight on the genetic structure of Sal populations in Bangladesh. Data obtained in the present study suggested that high level of genetic diversity was present in Sal population of Madhupur, followed by Cumilla and Dinajpur. To fight back the unescapable rate of overexploitation and deforestation of Sal forests, conservation interventions should be taken with new and developed *in situ* as well as *ex situ* strategies to preserve these genetic resources. In addition, isolated and fragmented Sal populations of Dinajpur also need proper attention and should be taken under suitable management.

Acknowledgements

The authors acknowledge the financial support given by the Ministry of Science and Technology, Government of the People's Republic of Bangladesh under the Special Allocation for Research and the authorities of the Department of Botany, University of Dhaka for providing logistics to conduct this piece of study during the year from 2017 to 2018.

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(Manuscript received on 14 March, 2020; revised on 23 November, 2020)