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GENETIC DIVERSITY AND POPULATION STRUCTURE OF *GREWIA NERVOSA* (LOUR.) PANIGRAHI FROM GAZIPUR AND CUMILLA SAL FORESTS, BANGLADESH

ASHFAQUE AHMED* AND SYDUL ISLAM

Ecology, Environment and Natural Resource Laboratory, Department of Botany, University of Dhaka, Dhaka -1000, Bangladesh

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Abstract

The study was carried out to determine the genetic diversity and population structure of a medicinal plant *Grewia nervosa* (Lour.) Panigrahi from two different regions of Sal forests, one from Cumilla showed more abundance than Sal plant and Gazipur showed relatively less abundance of it. Interest was taken to find out the best genotypes for breeding and conservation program. With these purposes 10 RAPD and 7 ISSR loci were used in the present investigation. Populations from Cumilla Sal forest showed significantly higher polymorphism and comparatively higher heterozygosity than that of Gazipur. Very low gene flow and high differentiation among the populations was noticed in Gazipur Sal forest whereas moderate gene flow and less differentiation of populations of Cumilla

Introduction

Grewia nervosa (Lour.) Panigrahi is a woody shrub or small tree belongs to the family Tiliaceae. It is a medicinal plant traditionally used to treat jaundice, cold, heat stroke, fever, diarrhea (Bandara *et al.* 2000, Kalita and Deb 2004, Luo *et al.* 2009). Owing to high medicinal background breeding and conservation program of this species should get emphasized. In view of this, study on genetic diversity of this species becomes a focal point as distributions of variation within and among populations formed the basis of breeding program (Namkoong 1984).

Grewia nervosa appears as the most abundant plant species in Kotbari and Rajeshpur Sal forests in Cumilla even it showed relatively more abundance than Sal plant (Ahmed *et al.* 2015). Contrariwise, this plant was relatively less abundant in Gazipur Sal forest. Genotypic richness has been reported to increases the abundance of species (Reusch *et al.* 2005). So how, why and in what extent the individuals and populations of Gazipur and Cumilla Sal forests differ in genetic level is an important question in conservation ecology. Genetic variation of individuals and genetic differentiation among populations have resulted in adaptation to the environment and population process (Zhang *et al.* 2019) and these processes include drift, migration, dispersal and gene flow (Holderegger *et al.* 2006, Zhang *et al.* 2019). Determining genetic diversity can be based on agronomic, morphological, biochemical, and molecular types of information (Mohammadi and Prasanna 2003, Sudré *et al.* 2007, Gonçalves *et al.* 2009, Ahmed *et al.* 2017). A molecular type includes the use of several molecular markers such as RAPD, ISSR, SSR, AFLP etc. These different types of molecular markers are also different as to their potentiality to detect differences between individuals, their cost, facilities required, and consistency, and replication of results (Schlötterer 2004, Schulman 2007). However, the combined use of different markers can provide more reliable information about genetic diversity rather than using only one marker, the expectation is that some errors or problems presented by a certain marker could be minimized using other markers (Demeke *et al.* 1997, Saker *et al.* 2005). So, using both primers can be expected to give more precise idea about the genetic divergence of *G. nervosa*.

*Author for correspondence: <aashfaque67.bot@du.ac.bd>; <aashfaque67@yahoo.com>.

Although the relationship between the plant species diversity and soil have been extensively studied by ecologists (Clark and Tilman 2008, Laliberte *et al.* 2013), very little focus has been given to study the to find out the relationship between genetic diversity of a focal species of a population and soil properties (Xu *et al.* 2016). Therefore, aims of the present study was to focus on to determine the genetic diversity and population structure of one of the most important medicinal plant *G. nervosa* growing in abundant in different populations of two different Sal forests of Bangladesh and to find out the effect of soil pH on the genetic diversity of this species.

Materials and Methods

At first, 15 leaf samples each from 5 different populations of Gazipur and Cumilla Sal forests were collected. Based on the quality and amount of DNA isolated, 29 individuals were used in the present investigation. Eleven and 18 samples were used from Gazipur and Cumilla Sal forest, respectively (Table 1).

DNA from each leaf sample was extracted following the method proposed by Doyle and Doyle (1987). To detect DNA polymorphism 10 RAPD and 7 ISSR primers were used in the present study (Table 2). Primers were selected based on the work of Verma (2015).

Table 1. **Populations of *Grewia nervosa* with region, co-ordinates, and sample size.**

Population	Region	Latitude	Longitude	Sample size
Nanduain	Gazipur	24°30′	90°23′	5
Chandra	Gazipur	24°20′	90°14′	6
Sribiddagram	Cumilla	23°43′	90°24′	6
Kotbari	Cumilla	23°25′	91°80′	6
Rajeshpur	Cumilla	23°21′	91°16′	6

Table 2. Name of primers along with the sequences, range of band amplification and melting temperature.

RAPD primers	Sequence (5'-3')	Length of amplified bands (bp)	Temp. (ºC)	ISSR primers	Sequence (5'-3')	Length of amplified bands (bp)	Temp. (ºC)
OPA- 04	AATCGGGCTG	250- 5000	30	810	GAGAGAGAGAGAGAGAT	220-1600	50
OPA- 05	AGGGGTCTTG	750- 4000	30	812	GAGAGAGAGAGAGAGAA	250- 1900	49
OPC- 04	CCGCATCTAC	800- 1200	30	825	CACACACACACACACC	300- 1200	48
OPC- 06	GAACGGACTC	200- 1200	30	835	AGAGAGAGAGAGAGAGYC	220- 1400	54
OPC- 13	AAGCCTCGTC	350- 2500	30	861	ACCACCACCACCACCACC	280- 1200	60
OPF- 06	GGGAATTCGG	500- 3000	30	862	AGCAGCAGCAGCAGCAGC	210- 700	61
OPF- 08	GGGATATCGG	250- 2100	30	UBC 827	ACACACACACACACACAG	250- 1400	54
OPF- 12	ACGGTACCAG	250- 3800	30				
OPF- 13	GGCTGCAGAA	270- 2900	30				
OPO- 20	ACACACGCTG	280- 2000	30				

PCR products obtained by using RAPD and ISSR primers were electrophoresed on 0.8 and 1.4 % agarose gel (stained with ethidium bromide), respectively. One kb and 100bp ladder were also electrophoresed alongside the PCR products. Gel electrophoresis was run for 50 and 60

minutes in case of RAPD and ISSR analysis, respectively. Amplified DNA fragments were visualized on UV-transilluminator and photographed by a Gel Documentation system (CSL-MicrodocSystem, Cleaver Scientific Ltd).

Band size was estimated by the comparison with 1 kb and 100 bp ladder and data was scored by following the presence (1) and absence (0) of bands. Gene diversity (h), Shannon information Index (I), observed number of allele (Na), effective number of allele (Ne) and percentage of polymorphism were calculated using POPGENE version 1.32.

Principal coordinate analysis (PCoA) and analysis of molecular variance (AMOVA) were performed by using the program GenAlEx version 6.1 to detect population structure and percentage of variance in the populations. Significance of variance components were tested on 9999 permutation. Mantel test was employed to find correlation present between genetic and geographic distance.

Analysis of the correlation between soil data (Propa *et al*. 2021) and molecular data was accomplished by using excel 10 and F test by using Minitab 14.

Results and Discussion

The selected ten primers used in the present study produced polymorphic banding patterns in the investigated samples. A total of 73 alleles were produced with an average of 7.3 alleles per locus using RAPD primers while total 64 alleles were scored with an average of 9.14 alleles per locus in case of ISSR primers. All loci showed 100 % polymorphism except two ISSR loci UBC-827 and 861. In average 63.56 and 69.37 % polymorphism were observed for the five populations, respectively in RAPD and ISSR analysis. The gene diversity h value for 5 investigated populations ranged from 0.184 to 0.315 in RAPD primer and 0.215 to 0.328 in ISSR primer-based analysis (Table 3). Highest values of h were 0.315 for Kotbari and 0.328 for Rajeshpur population in RAPD and ISSR analysis, respectively.

Table 3. Genetic variability of *Grewia nervosa* in five different populations.

Population	Na	Ne	h	I	PPB	PAL
RAPD						
Nanduain	1.424	1.336	0.184	0.264	42.47	3.2
Chandra	1.561	1.317	0.194	0.295	56.16	4.0
Sribiddagram	1.684	1.475	0.272	0.399	68.49	5.1
Kotbari	1.708	1.552	**0.315**	0.460	**78.08**	**5.6**
Rajeshpur	1.726	1.488	0.281	0.415	72.60	5.4
ISSR						
Nanduain	1.578	1.356	0.215	0.321	57.81	3.6
Chandra	1.578	1.400	0.230	0.337	57.81	3.7
Sribiddagram	1.718	1.505	0.286	0.419	71.88	4.6
Kotbari	1.734	1.512	0.293	0.430	73.44	4.7
Rajeshpur	1.843	1.569	**0.328**	0.483	**84.38**	**5.4**

Na: Observed number of allele, Ne: Effective number of allele, h: Nei's (1973) gene diversity, I: Shannon's information index, PPB: Percentage of polymorphic band and PAL: Polymorphic allele per locus.

The gene diversity (h) value and Shannon Information Index (I) were considerably higher in Cumilla Sal Forest (Table 4). Alongside Cumilla region showed significantly higher number of polymorphic alleles per locus (RAPD- 5.366 and ISSR- 4.90) compared to that of Gazipur region (RAPD- 3.60 and ISSR- 3.65).

Table 4. Genetic variability of *Grewia nervosa* in Gazipur and Cumilla region.

Region	Na	Ne	h	I	PPB	PAL
RAPD						
Gazipur	1.492	1.326	**0.189**	0.279	**49.315**	**3.600**
Cumilla	1.706	1.505	**0.289**	0.424	**73.056**	**5.366**
ISSR						
Gazipur	1.578	1.378	**0.222**	0.329	**57.810**	**3.650**
Cumilla	1.765	1.528	**0.302**	0.444	**76.566**	**4.900**

Elaboration of abbreviations are same as Table 3.

A positive and significant correlation was found between the heterozygosity and pH of soil (P = 0.004 in RAPD and P = 0.002 in ISSR) but not between the heterozygosity and soil moisture content. It was found that pH and heterozygosity were higher in Cumilla compared to that of Gazipur Sal forest (Fig. 1). The pH and moisture content values of soil were collected from Propa *et al.* (2021).

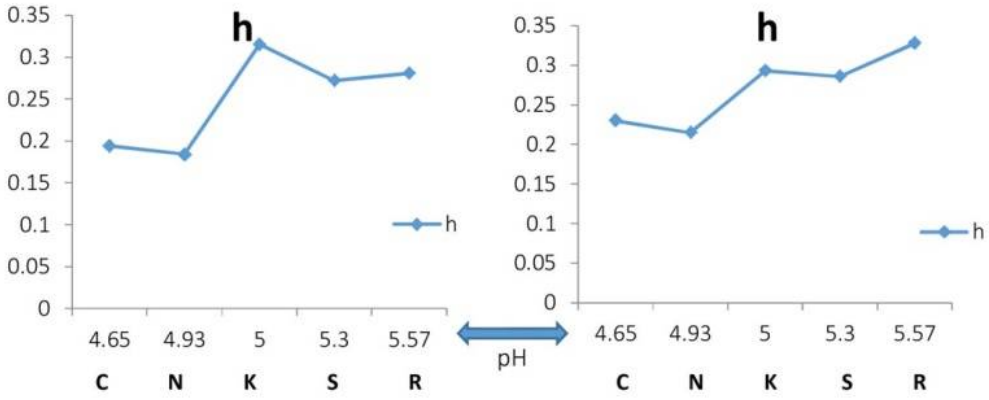

Fig 1. Correlation between heterozygosity (h) and soil pH, left one for RAPD and right one for ISSR data (C = Chandra, N = Nanduain, K = Kotbari, S = Shribiddagram, R = Rajeshpur).

Mantel test showed less but positive and significant isolation by distance for both primers r = 0.235, P = 0.0001 for RAPD; r = 0.0557, P = 0.001 for ISSR, which indicates significant isolation by distance existed among the populations at a very less extent.

Analysis of molecular variance (AMOVA) was employed to detect the distribution of variance in the populations, which revealed largest part of total variations was within populations, 67 % (P = 0.0001) and 77 % (P = 0.0001) in RAPD and ISSR analysis, respectively (Table 5).

AMOVA test showed significant differentiation among the populations and among the regions for both primers.

Table 5. Analysis of molecular variance of five *Grewia nervosa* populations.

Source	df	SS	MS	Estimate of Variation	Percentage	P
RAPD						
Among regions	1	54.511	54.511	1.536	9	0.002
Among Pops	3	101.774	33.925	3.924	24	0.0001
Within Pops	24	266.267	11.094	11.094	67	0.0001
ISSR						
Among regions	1	36.464	36.464	1.055	8	0.007
Among Pops	3	66.797	22.266	2.011	15	0.0001
Within Pops	24	253.567	10.565	10.565	77	0.0001

df: Degree of freedom, SS: Sum of square, MS: Mean of square, P: Probability level.

The first two coordinates of principal coordinate analysis (PCoA) explained 49.09 and 35.99 % of the variation for RAPD data set, respectively while in ISSR analysis first axis explained 57.85 % and second axis explained 26.52 % of total variation. Principal coordinate analysis formed clusters and separated populations reflecting geographical distribution. Principal coordinate analysis propounded that 2 populations from Gazipur Sal forest showed greater genetic distance among them while 3 populations from Cumilla formed a cluster (Fig. 2).

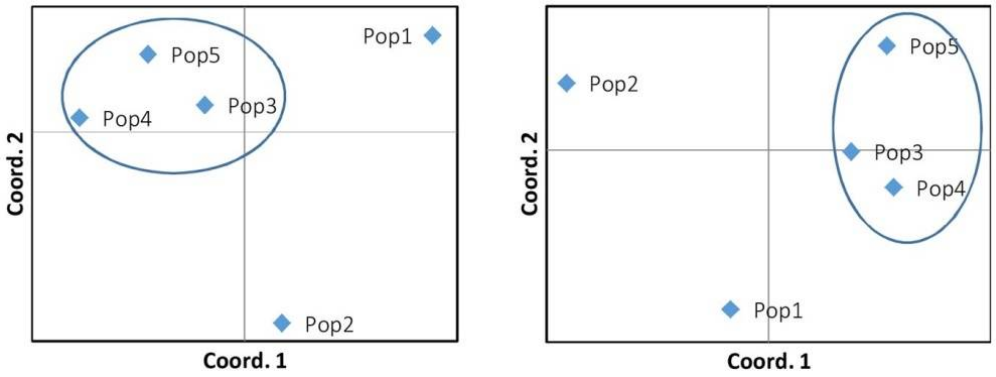

Fig. 2. Principal coordinate analysis of five populations based on genetic distance obtained from 10 RAPD loci (left) and 7 ISSR loci (right). Pop1 = Nanduain, Pop2 = Chandra, Pop3 = Shribiddagram, Pop4 = Kotbari, Pop5 = Rajeshpur.

Pairwise population matrix of Nei genetic distance was obtained by analyzing the combined data of RAPD and ISSR primers (Table 6). Highest genetic differentiation (0.290) was found between the populations Chandra and Kotbari. Populations from Cumilla Sal forest showed relatively less genetic differentiation among them. Interestingly, Nanduain and Chandra populations from Gazipur showed high differentiation (0.275) among them (Table 6).

Table 6. Pairwise Nei genetic distance (below diagonal) of five populations of *Grewia nervosa.*

Nanduain	Chandra	Sribiddagram	Kotbari	Rajeshpur	Populations
0.000					Nanduain
0.275	0.000				Chandra
0.203	0.249	0.000			Sribiddagram
0.271	**0.290**	**0.114**	0.000		Kotbari
0.273	0.292	**0.177**	**0.144**	0.000	Rajeshpur

Pair wise PhiPT values were obtained from the combined data set of RAPD and ISSR primers (Table 7). Though Nanduain and Chandra populations are from the same geographical area, these two showed highest PhiPT value of 0.320 (Table 7). PhiPT value for 3 populations of Cumilla Sal forest ranged from 0.120 to 0.233, with lowest PhiPT value of 0.120 observed between Shribiddagram and Kotbari populations.

Table 7. Pairwise population PhiPT values (below diagonal) and probability, P (rand > = data) based on 9999 permutations (above diagonal).

Nanduain	Chandra	Sribiddagram	Kotbari	Rajeshpur	Populations
0.000	0.001	0.004	0.001	0.003	Nanduain
0.320	0.000	0.003	0.002	0.002	Chandra
0.213	0.275	0.000	0.015	0.002	Sribiddagram
0.314	0.313	**0.120**	0.000	0.002	Kotbari
0.318	0.318	**0.233**	**0.141**	0.000	Rajeshpur

From the analysis with two different types of dominant loci RAPD and ISSR demonstrated high level of polymorphism in different populations of *G. nervosa.* On average, ISSR loci (69.06 %) were more polymorphic than RAPD loci (63.56 %). Population of Kotbari (78.08%) and Rajeshpur (84.38 %) were found with the highest polymorphism rate among all the populations respectively in RAPD and ISSR analysis.

Genotypic richness increases the abundance of species (Reusch *et al.* 2005). RAPD data gave mean h values of 0.189 ± 0.203 in Gazipur and 0.289 ± 0.192 in Cumilla, while the mean h values in Gazipur and Cumilla were 0.222 ± 0.200 and 0.302 ± 0.185, respectively for ISSR data. Analysis based on these two molecular markers revealed that there is no significant difference in Nei within population diversity (h) of *Grewia nervosa* between Gazipur and Cumilla Sal forest. But rate of polymorphism and number of polymorphic allele per locus (P = 0.015 in RAPD, P = 0.03 in ISSR) were significantly higher in Cumilla Sal forest. *Grewia nervosa* was more abundant in Cumilla Sal forest where it showed significantly higher polymorphism and considerably higher within population variation (h).

Although a positive correlation between heterozygosity and soil pH was found in this study, other study showed a negative correlation. Xu *et al.* (2016) have studied the effect of environmental properties on the genetic diversity of *Beilschmiedia roxburghiana* (Lauraceae) in the tropical seasonal rainforest in Xishuangbanna, south-western China and found that soil pH and phosphorus had significantly decreased the genetic diversity of *B. roxburghiana*.

Analysis of Molecular variance (AMOVA) confirmed about the significant differentiation between Gazipur and Cumilla region (9 % and 8 % of total variation respectively in RAPD and ISSR analysis). The largest part of total variation was within the population (RAPD- 67%, P = 0.002 and ISSR- 78 %, P = 0.007) and significant correlation between genetic and geographic distance revealed by mantel test suggested that no occurrence of genetic drift in all the 5 populations of *G. nervosa*.

The *G. nervosa* populations of Nanduain and Chandra areas from Gazipur Sal forest showed high genetic differentiation among them though geographically these two populations were closer. High genetic distance (0.275) was found in between these two indicating high differentiation which was further supported by high PhiPT value (0.320). High PhiPT value leads to the assumption about a very low level of gene flow between these two populations. Low level of gene flow and high level of inter population differentiation could be a reflection of limited seed and pollen dispersal and inbreeding nature of plant (Surabhi *et al.* 2017). On the other hand, PhiPT value between the populations of Gazipur and Cumilla ranged from 0.213 to 0.318 indicating low gene flow between the populations of Gazipur and Cumilla Sal forest. So, the evidence of low gene flow among the populations of Gazipur and Cumilla as well as between the populations of Gazipur suggested the isolation of Gazipur populations from Cumilla populations and the fragmentation of the populations of Gazipur Sal forest. Population fragmentation can lead to inbreeding and loss of genetic diversity within fragments (Frankham *et al.* 2002). So, lower gene diversity (h value for RAPD- 0.189 and ISSR- 0.222) and high population differentiation suggested about the inbreeding nature. It also indicated self-pollination is more frequent in these two populations over out crossing. Plant-animal mutualism such as pollination and seed dispersal are crucial for maintaining gene flow and population dynamics in plant populations (Slatkin 1985, Herrera and Pellmyr 2002). Lack of pollinator can facilitate selfing and low gene flow between populations and increase differentiation.

Genetic distance between the 3 populations of Cumilla Sal forest ranged from 0.114 to 0.177 indicating comparatively lower differentiation. PhiPT value of these populations ranged from 0.120 to 0.233, with the lowest PhiPT value (0.120) between the populations of Shribiddagram and Kotbari. So, gene flow found to be at a moderate level among the populations of Cumilla Sal forest. High Nei within population variation (h value) in Cumilla (RAPD- 0.289 and ISSR- 0.302), moderate level of gene flow and low population differentiation could be due to high pollen flow, seed dispersal and a moderate level of out crossing over selfing. As *G. nervosa* was more abundant in Cumilla Sal forest so the availability for pollination was also higher that can facilitate out crossing.

Information on genetic diversity and relationship among populations is important for plant breeding programs, as it helps to select the right genetic material to be used (Ganesh and Thangavelu 1995), and the distributions of variation within and among populations formed the basis of breeding program (Namkoong 1984). Individuals from Kotbari and Rajeshpur showed highest heterozygosity and polymorphism. Heterozygosity and fitness are significantly correlated with each other (Reed and Frankham 2003). So, individuals from these two populations assumed to be the fittest and more genetically diverse. If breeding is a concern, then individuals from these 2 populations should keep at the best priority level.

This study seems to be the first comprehensive study on genetic diversity analysis of *G. nervosa*. It was more abundant in Cumilla Sal forest and the populations of *G. nervosa* in Cumilla Sal forest showed significantly higher polymorphism and considerably higher heterozygosity than that of Gazipur Sal forest. High differentiation and lower heterozygosity of the populations of Gazipur Sal forest can be the consequence of low gene flow and more frequently happening

inbreeding system. Conservation should be a matter of concern in Gazipur Sal forest to avoid further reduction of population size due to inbreeding depression and population fragmentation.

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