Genetic diversity analysis in *Gaultheria fragrantissima* Wall. (Ericaceae) from the two biodiversity hotspots in India using ISSR markers

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Our study involves Inter Simple Sequence Repeat markers to analyse genetic diversity of an undershrub, *Gaultheria fragrantissima* Wall. (Ericaceae) from two plant diversity hotspots in India, namely Western Ghats and Northeastern Himalayas. The plants from these two regions show no morphological differences but the total heterozygosity (H_T = 0.505) is high. Furthermore, the average heterozygosity of *G. fragrantissima* at WG (H_s = 0.433) is higher than that at NE region (H_s = 0.231). Within population variance is higher (15.56%) than among population variance (8.31%) as seen in outcrossing plant species. Mantel's test shows a strong positive correlation between the genetic and geographic distances. The plants within WG show high gene flow, which may be enough to prevent genetic drift, however, the gene flow in NE population is very low.

Keywords: *Gaultheria fragrantissima*, genetic diversity, heterozygosity, ISSR markers.

STUDIES on genetic diversity and analysis of genetic structure of plant populations are important to understand various complex processes involved in long-term evolutionary history of the species such as genetic drift, gene flow, mutation and selection[1,2]. Most of such studies have been carried out using allozymes[3–5] and RAPD markers[6–8]. ISSR markers have also exhibited great potential in such studies of natural populations in addition to their demonstrable utility in analysis of cultivated species[9–11]. They have been used to study genetic diversity in natural populations of *Primula obconica*[12], genetic diversity of endangered *Monimopetalum chinense*[13] and *Ammopiptanthus* populations[14], intra- and inter-species genetic variations in three montane plant species from India[15] and to examine patterns of hybridization and hybrid speciation in *Penstemons*[16].

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Gaultheria fragrantissima Wall. is an outcrossing insect-pollinated plant with chromosome number n = 22, growing at the fringes of shola forest or slopes above 1500 m altitude[17]. It is a medicinally important plant and oil extracted from its leaves is popularly known as 'Oil of Indian wintergreen'. The oil contains methyl salicylate as the chief constituent, which is prescribed for rheumatic arthritis, sciatica, and neuralgia and is also used in most of the proprietary balms, liniments or ointments[18]. It occurs only in the two plant diversity hotspots in India, viz. Western Ghats (WG), also known as the Malabar rainforest and the Northeastern Himalayas (NE). *G. fragrantissima* forms a group of plant species along with *Rhododendron arboreum*, *Eurya nitida*, *Symplocos laurina*, *Mahonia leschnaultii*, etc. that are common to both the regions.

Many theories have been put forth by various researchers to explain the geographical distribution and migration of plant species between the NE and WG mountain regions. The most commonly held view for the occurrence of such species observed in NE and WG is that the populations are relicts or vestiges of the last glaciation[19] during the Pleistocene, which pushed the Himalayan plants southwards[20]. In contrast, Blasco[21] suggests the distribution to be due to long distance dispersal by birds or wind.

In our laboratory, we have been interested in using various molecular approaches to study the genetic diversity of specific montane plants that are common to these two plant diversity hotspots in India. As the first step, 12 plants of *Gaultheria fragrantissima*, 9 plants of *Eurya nitida* and 15 plants of *Symplocos laurina* were collected from WG and analysed using ISSR markers[15]. In the present study we have included 66 plants of *Gaultheria fragrantissima* from WG and NE regions for the ISSR analysis. Such efforts are expected not only to give an insight into the gene flow, genetic drift, and the role of physical barriers, but are also useful in designing conservation strategies and assessing different theories on migration and evolution.

Materials and methods

Plant material

A total of 66 plants from seven populations representing the WG and NE regions were collected. The details of sampling are given in Table 1. The number of plants per population ranged from 5 to 16 and among population distances were not less than 10 km as suggested by Nybom and Bartish[22]. In fact, the minimum aerial distance observed among populations from Naduvattum and Avalanche was about 14 km while the maximum distance was 2325 km among the populations from Munnar and Shillong.

Three populations were collected from the Nilgiri hills, namely Naduvattum, Avalanche and Kotagiri road while Kodaikanal and Munnar were represented by one population each. In the NE hotspot, Cherapunji road and Shillong peak were the two populations representing Meghalaya state. The sample collection strategy and tours were planned and executed along with the Botanical Survey of India (BSI). Prior to collections, the herbarium records of BSI were referred for the identification of collection sites. However, some of these sites were observed to be cleared of its vegetation for human settlements or other human activities during the actual sampling. Secondly, at some of the sites, even after extensive exploration, we could sample only limited number of plants.

DNA isolation, PCR amplification and data analysis

Isolation of DNA and PCR amplification using ISSR primers were performed as described previously[15].

To avoid bias in parameter estimation, analysis of population genetic structure was restricted to those ISSR loci that fulfilled the $3/N$ criterion (observed frequencies were less than $1 - [3/N]$) where N is the number of plants[23]. Assuming that all populations are in Hardy-Weinberg equilibrium, the frequency of the null allele at locus $i(q_i)$ was estimated as $q_i = [x_i]^{1/2}$ (ref. 23). Other parameters were computed like percentage polymorphism (P), Nei's[24,25] measures of heterozygosity which includes total heterozygosity, H_T, with Lynch and Milligan[23] correction, $H_T = 2q_i(1 - q_i) + \mathrm{Var}(q_i)$ where q_i is the frequency of the null allele at ith locus in a population, mean heterozygosity within a population (H_s), diversity among populations ($D_{ST} = H_T - H_S$) and the coefficient of population differentiation ($G_{ST} = D_{ST}/H_T$). Gene flow (N_m) or the number of migrants entering a population in each generation was estimated indirectly using Wright's formula[26], $N_m = (1 - F_{ST}/4F_{ST})$, where F_{ST} is the standardized variance among populations and is considered equivalent to G_{ST} (ref. 25).

To illustrate relatedness among different individuals and among populations, the presence–absence matrix of ISSR bands was analysed using cluster analyses based on the unweighted pair group method with arithmetic mean (UPGMA). Dendrogram was constructed with the help of the commercial software package Winboot[27] using Jaccard coefficient. Pairwise similarity was calculated using Windist programme[27].

AMOVA (Analyses of Molecular Variance)[28] was used to partition the variance between main geographic regions, among populations and among individuals within populations using the program WIN AMOVA 1.55 provided by Excoffier *et al.* (http://anthropologie.unige.ch/ftp/ comp).

Distance matrix was employed for PCO (Principal Co-ordinate) analysis, which was performed with the help of NTSYS-PC program version 1.8 (ref. 29). The Mantel's test[30] was applied for correlations between the matrix of genetic diversity and spatial distance. Geographic distance matrix was constructed using the distances among the seven populations in kilometers and genetic distance matrix was constructed using the average genetic distances among the seven populations.

Results

Divergence between populations of G. fragrantissima

In our previous study[15], 32 ISSR primers were identified to be suitable for genetic diversity analysis of *G. fragrantissima* based on 12 samples. Of these, 17 ISSR primers that were polymorphic and gave good banding pattern were selected for the present work. As seen from Table 1, 47 and 19 samples represented the WG and NE

Table 1. Details of populations of *G. fragrantissima*

Region	Area of collection	No. of plants	Latitude	Longitude	Altitude (m)
WG	Naduvattum	5	11°28′60 N	76°52′60 E	1952
	Avalanche	16	11°22′0 N	76°31′00 E	2147
	Kotagiri road	10	11°25′60 N	76°52′60 E	1792
	Kodaikanal	10	10°13′0 N	77°28′60 E	1966
	Munnar	6	10°5′60 N	77°04′00 E	1603
NE	Cherapunji road	6	25°26′928 N	91°49′10 E	1485
	Shillong peak	13	25°34′0 N	91°52′60 E	1525

WG, Western Ghats, NE, Northeastern Himalayas.

Himalayan collection of *G. fragrantissima* respectively. The 17 ISSR primers amplified 129 loci in 66 individual samples of *G. fragrantissima*, of which 112 loci were polymorphic (86.82%) while 17 loci representing 13.17% were common to the two regions (Table 2). Each primer amplified 2 to 12 bands with a mean of 7.5 in the range of 200 to 1500 bp, among which 22 from WG and 21 from NE were observed to be unique bands. $(AG)_8$YA repeat amplified maximum number of polymorphic loci (12) and a minimum of 2 were amplified by $(TC)_8$RT (Table 2). Similarity index within population ranged from a maximum of 0.990 (Gf9A3 and Gf9A1) to a minimum of 0.33 (Gf4J11 and Gf9B6; Gf9B1; Gf9A5) (Table 3).

The diversity within populations of *G. fragrantissima* was assessed using various parameters. As given in Table 4, the total heterozygosity (H_T) was 0.505 while average heterozygosity (H_S) was in the range of 0.147–0.380. The range of variation for other parameters namely, average gene diversity within populations (D_{ST}), proportion of genetic diversity (G_{ST}) and gene flow (N_m) was observed to be 0.125–0.357, 0.247–0.708 and 0.103–0.759, respectively. Gene flow (N_m) within WG was 1.51 while within NE region was 0.210 (Table 4).

AMOVA analysis and cluster analysis

The details of the AMOVA have been depicted in Table 5. A major variation of the order of 76.13% was found between the two main distant regions (WG and NE) by

Table 2. Genetic diversity revealed by various repeat motifs in *G. fragrantissima*

Repeat	Anchor	Monomorphic loci	Polymorphic loci	Percentage polymorphism
$(CT)_8$	A	2	6	75.0
$(CT)_8$	G	0	6	100.0
$(CT)_8$	RC*	1	10	90.9
$(CT)_8$	RG	1	7	87.5
$(CT)_8$	T	0	8	100.0
Mean				90.68
$(TC)_8$	G	1	6	85.7
$(TC)_8$	RA	1	6	85.7
$(TC)_8$	RG	0	3	100.0
$(TC)_8$	RT	2	2	50.0
Mean				80.35
$(AG)_8$	YT**	0	8	100.0
$(AG)_8$	YC	2	8	80.0
$(AG)_8$	YA	0	12	100.0
Mean				93.33
$(AC)_8$	YT	2	5	71.4
$(AC)_8$	YA	1	7	87.5
Mean				79.45
$(GA)_8$	C	3	6	66.6
$(GA)_8$	T	0	9	100.0
Mean				83.3
$(CA)_8$	G	1	3	75.0
Total		17	112	86.82

*R, A/G; **Y, C/T.

Table 3. Similarity index within different populations *G. fragrantissima*

Population	Maximum	Minimum
Naduvattum	0.952 (Gf4H4and Gf4C7)	0.882 (Gf4C6; Gf4G8 and Gf4A1)
Avalanche	0.947 (Gf4J27 and Gf4J25)	0.794 (Gf4J30 and Gf4J11)
Kotagiri road	0.970 (Gf4M14 and Gf4M13)	0.873 (Gf4M5 and Gf4M15)
Kodaikanal	0.966 (Gf7E18 and Gf7E17)	0.83 (Gf7E21and Gf7E13)
Munnar	0.927 (Gf12A7 and Gf12A3)	0.793 (Gf12A3 and Gf12A1)
Cherapunji road	0.990 (Gf9A1 and Gf9A3)	0.930 (Gf9A2 and Gf9A6)
Shillong peak	0.989 (Gf9B9and Gf9B11)	0.877 (Gf9B7 and Gf9B1)
WG–NE (all 66 plants)	0.458 (Gf4M11 and Gf9B5)	0.33 (Gf4J11 and Gf9B6; Gf9B1; Gf9A5)

Gf, *Gaultheria fragrantissima*, the number after Gf indicates the number given to the location, namely 4, Nilgiri Hills (Naduvattam, Avalanche and Kotagiri road); 7, Kodaikanal; 9, Shillong (Cherapunji and Shillong peak) and 12, Munnar, the alphabet after the number indicates the name of a specific site in the respective population followed by the number of the plant.

Table 4. Various parameters of diversity within populations of *G. fragrantissima*

Population	H_S	D_{ST}	G_{ST}	N_m
Naduvattum	0.277	0.228	0.451	0.304
Avalanche	0.362	0.143	0.283	0.632
Kotagiri road	0.305	0.200	0.396	0.380
Kodaikanal	0.380	0.125	0.247	0.759
Munnar	0.359	0.146	0.289	0.613
Cherapunji road	0.147	0.357	0.708	0.103
Shillong peak	0.222	0.283	0.560	0.195
WG	0.433	0.072	0.142	1.51
NE	0.231	0.274	0.542	0.210
Total	0.505 (H_T)	–	–	–

H_S, Average heterozygosity; H_T, Total heterozygosity; D_{ST}, Average gene diversity within populations; G_{ST} Proportion of genetic diversity; N_m, Gene flow. The formulae to calculate these values are described in materials and methods.

AMOVA. It revealed 15.56% variance within the population when all the 66 individual samples were considered, which was higher than 8.31% variance among the seven populations. Cluster analysis was performed using UPGMA algorithms as well as the PCO approach and dendrogram was drawn using Jaccard coefficient. Jaccard coefficient does not incorporate shared band absences, which is not desirable because a band absence may not always indicate a homozygous recessive genotype. Instead, it may reflect loss of a primer binding site, or insertions/deletions within a fragment. Figure 1 shows two distinct groups representing NE and WG populations. Within WG the three populations from Nilgiri hills (Naduvattum, Avalanche and Kotagiri road) formed one group and those from Kodaikanal and Munnar formed the second group though some individuals from Kodaikanal and Munnar formed a closer group. There was one exception where Gf4J30 from Avalanche grouped more closely with Kodaikanal and Munnar group (Figure 1). PCO analysis revealed the same distribution pattern of the population shown by the dendrogram and confirmed the groups (figure not shown). The Mantel's correlogram calculated for genetic distance and geographic distance for *G. fragrantissima* revealed a strong positive correlation between them ($r = 0.994$; $P < 0.01$).

Discussion

Genetic diversity within and between populations of G. fragrantissima

India is one of the 12 megadiversity centres of the world, and Western Ghats and Northeastern Himalayas (Indo-Burma region) constitute two of the 25 biodiversity hotspots of the world[31]. The two hotspots harbour a high percentage of endemic plants, rich biodiversity and also higher number of rare and threatened taxa. The country also lies at the junction of the three major biogeographic realms, namely, the Indo-Malayan, the Eurasian and the Afro-tropical. The two regions under study, viz. WG and NE, have been marked for the overall national strategy of protecting the ecosystems and safeguarding the genetic diversity. India is a developing country where threats are the greatest and conservation resources are the most scarce[32]. Therefore, efforts are needed especially in India, to conserve and maintain genes, species and ecosystems for the sustainable use and management of biological resources.

In the light of above scenario, genetic diversity-directed conservation would be a better proposition to be adopted in a country like India. The present study has revealed the status of genetic diversity present in *G. fragrantissima* in India. The dendrogram clustered the individuals of *G. fragrantissima* in distinct groups according to their geographical origin. Two major clusters were formed, each grouping individuals from WG and NE regions. Plants from WG showed genetic similarity in the range of 0.794 to 0.970 while those from NE showed a narrow range between 0.877 and 0.990. On an average, plants from WG grouped according to their specific geographic location, with a few exceptions. The narrow range of similarity index values seen in NE as compared to WG was in accordance with the low average heterozygosity value of 0.231 in NE plants. The PCO analysis (figure not shown) showed similar clustering as that in the dendrogram supporting the grouping of individuals. Significant positive correlations between genetic and geographic distances using Mantel's test ($r = 0.994$, $P < 0.01$) also supported the dendrogram and PCO analysis.

Since *G. fragrantissima* is an outcrossing plant species, within population genetic diversity was calculated assuming the Hardy–Weinberg equilibrium. The total heterozygosity was observed to be 0.505 when all 66 individuals from the two main regions (WG and NE) were considered together. This suggests that there is genetic variation within *G. fragrantissima* though the plants from WG and NE regions do not show any distinct morphological variation (personal observation). The value of H_T in *G. fragrantissima* was also high as compared to those reported earlier (0.238 in *S. laurina*[15], and 0.290 in *Pueraria lobata* an introduced clonal invasive plant[5]). However, in case of individual populations, the average heterozygosity (H_S) for NE region populations was low as compared to H_S of WG (Table 4). According to Frankel *et al.*[33], higher the heterozygosity better is the fitness of populations, whereas lower the heterozygosity less is the population viability. Thus the plants from NE region may be genetically more homogenous as compared to the plants from the populations of WG. The G_{ST} values of the different populations of WG (Table 4) were in accordance with those reported for outcrossing plant species [0.1–0.24 (ref. 34); 0.099–0.216 (ref. 35) and 0.03–0.31 (ref. 36)], while for the NE region G_{ST} value was higher (Table 4). Though G_{ST} is developed for codominant data, according to Nybom and Bartish[22], AMOVA derived ϕ_{ST} and Nei's G_{ST} when cal-

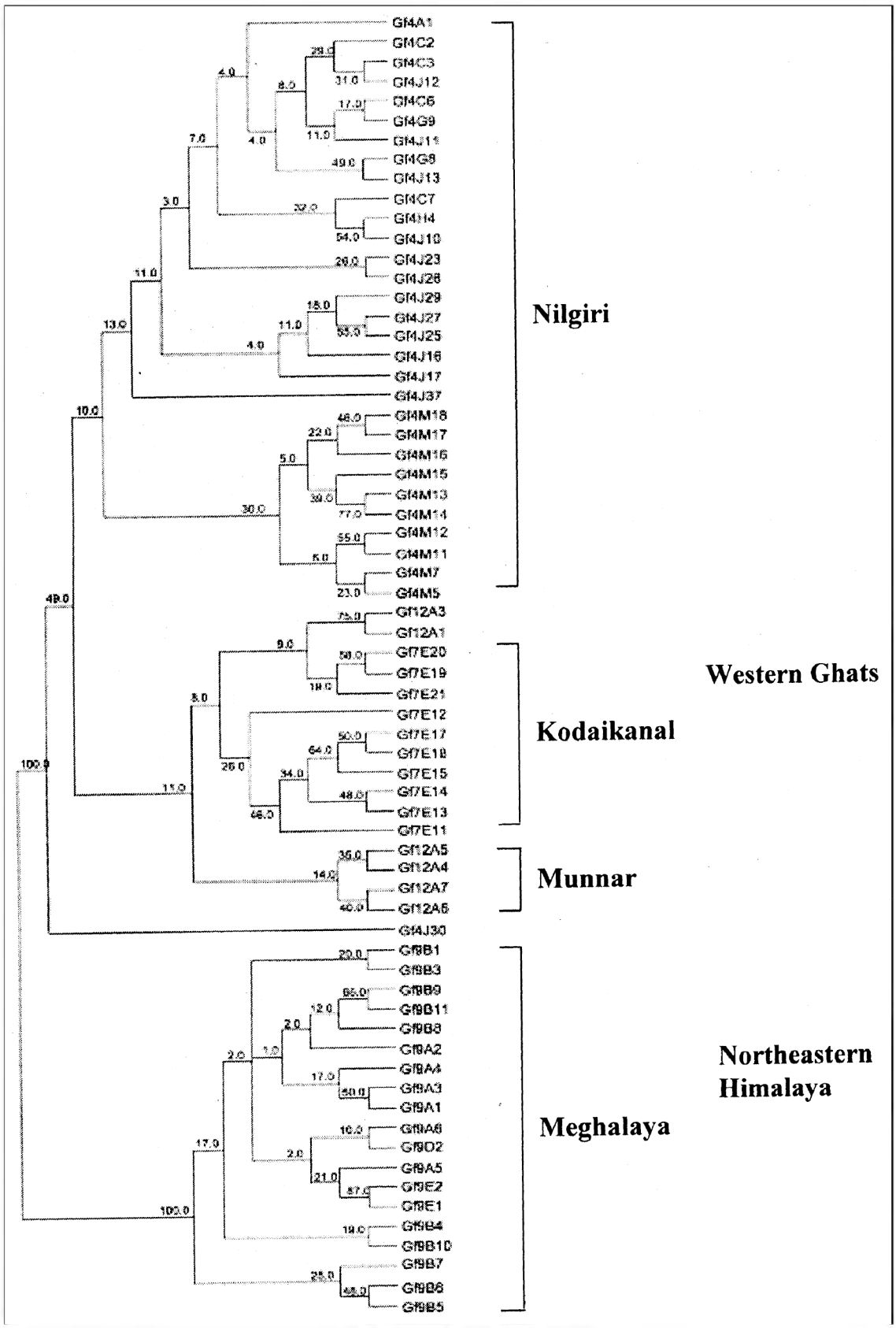

Figure 1. UPGMA dendrogram of *Gaultheria fragrantissima* populations from WG and NE regions in India. Gf, *Gaultheria fragrantissima*, the number after Gf indicates the number given to the location, namely, 4, Nilgiri hills (Naduvattum, Avalanche, Kotagiri road); 7, Kodaikanal; 12, Munnar (populations from Western Ghats) and 9, Meghalaya (Shillong peak, Cherapunji road): (populations from Northeastern Himalaya), the alphabet after the number indicates the name of the site in the respective location followed by the number of the plant.

Table 5. Analysis of molecular variance (AMOVA) for the 66 individuals sampled from 7 populations of *G. fragrantissima*

Source of variation	d.f.	SSD	MSD	VC	% total variance	*P*-value
Among regions (WG and NE)	1	810.24	810.24	28.47	76.13	< 0.001
Among populations	5	166.60	33.32	3.11	8.31	< 0.001
Within population	59	343.38	5.82	5.82	15.56	< 0.001

d.f., Degree of freedom; SSD, Sum of squared deviation; MSD, Mean squared deviation; VC, Variance component; % Total variance (percentage of total variance contributed by each component); *P*-value, Probability of obtaining a more extreme component estimate by chance alone.

culated for dominant data give estimates which are similar in nature to those obtained with codominant data. Populations from WG showed lower G_{ST} value (Table 4) than the populations from NE, indicating that the population differentiation was less in the WG populations. This observation suggests that the mechanism of seed dispersal and pollen flow (nuclear DNA is biparentally inherited) is probably efficient in WG. Populations from NE region, on the other hand, showed high G_{ST} value. However, in order to comment on the efficiency of seed dispersal and pollen flow we need to have more populations from NE region as only two populations have been analysed in the present work. Low G_{ST} value indicated high within-population genetic diversity which was also revealed by AMOVA where within-population variance (15.56%, $P < 0.001$) was higher than between them (8.31%, $P < 0.001$) indicating a relatively restricted population differentiation as expected in outcrossing species[37,38]. According to Hamrick and Godt[35], outcrossing species tend to have 10–20% of the genetic variation among populations while selfing species present, on an average, 50% of this variation among populations. In this study, among population variance, as calculated by AMOVA, was 8.31%, which was in accordance with the other reported values (13% in *Grevillea scapigera*[39], and 12% in *Cordia alliodora*, a highly outcrossing species[40]).

Gene flow within populations of G. fragrantissima

Table 4 gives the estimate of gene flow in the populations of *G. fragrantissima*. Gene flow within WG was 1.51 which might probably be good enough to prevent genetic drift indicating exchange of genes among the WG populations. The value of gene flow was much lower (0.210) within NE region (Table 4). The gene flow between the two hotspots was also low (0.480). Distance acts as a barrier between these two regions for exchange of genes. This probably suggests step by step migration of *G. fragrantissima* in the past and not long distance dispersal as suggested by Blasco[21] earlier. Levels of gene flow among populations of many plant species are low[41,42], which may allow differentiation among populations further leading to speciation. Such differentiation may be related to geographic distance among populations. In isolated populations, genetic drift may eventually reduce genetic variation as seen in the present study in *G. fragrantissima* populations at NE region. Such changes in genetic variability because of habitat fragmentation are relevant from plant conservation point of view since they may affect the plant fitness[43,44].

In summary, some of the important observations in this study indicate that *G. fragrantissima* plants from NE region with low average heterozygosity are genetically similar to each other. Secondly, the plants within WG show high gene flow, which may be enough to prevent genetic drift. However, the gene flow in NE population is very low.

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