Genetic diversity among spatially isolated populations of *Eurya nitida* Korth. (Theaceae) based on inter-simple sequence repeats

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In India, *Eurya nitida* occurs in the forests of the northeastern (NE) Himalayas and the Western Ghats (WG). Thirty-seven individuals from NE and WG populations were collected and analysed for genetic diversity profile using sixteen inter-simple sequence repeat markers. These individuals produced 95.45% polymorphic banding profiles. Total heterozygosity (H_T) and interpopulation heterozygosity (H_S) were 0.2665 and 0.1346 ± 0.059 respectively, while AMOVA yielded a moderate value of 35.20% across the locations. Low gene flow, UPGMA phenogram and Principal Co-Ordinate analysis revealed strong geographical structuring within the locations. These results impose a strong need to plan aggressive conservation strategies for *E. nitida*.

RECENTLY, 25 biodiversity hotspots have been reported from the world, which are the sole habitats of 44% of the earth's plant species[1]. Among these, eight are known to be areas of highest diversity and India is privileged to have two of them. The Indo-Burma region [including the northeastern region (NE) and western Himalayas from the Indian subcontinent] has nearly 7000 endemic plant species, which account for 2.3% of the total described plant species of the world. On the other hand, the Western Ghats (WG) and Sri Lanka have 2180 endemic plant species accounting for 0.7% of the world's total plant species[1]. In the Himalayan ranges, vegetation can be divided into three broad zones: tropical–subtropical (base to 1200 m), temperate (1200 to 3600 m) and alpine (up to 4500 m)[2]. The WG comprise a chain of mountain ranges that run along the western coast of India (Figure 1) and consist of hills whose elevations generally exceed 1000 m. The types of forest in this region include thorn forests (300 to 800 m), deciduous forests (800 to 1300 m) and tropical evergreen montane forests (above 1500 m)[3].

The evergreen forests of the NE and WG are more than 2000 km apart, not connected by evergreen forest ranges[4]. However, they are linked by phytogeograhic affinities[5]. These two montane regions have some common flora and fauna that include plant species such as *Rhododendron arboreum*, *Gaultheria fragrantissima*, *Eurya nitida*, *Symplocos laurina*, *Mahonia leshenaultii*, etc., which belong to the shrub savanna or the shola forest[6]. The most commonly held view for the occurrence of these common species is that the Pleistocene glaciation is responsible for pushing these Himalayan plants southwards[7], whereas according to Blasco[8,9], it is due to long-range dispersal by birds or wind. Meher-Homji[3] has pointed out certain problems with long-distance dispersal and has proposed three hypotheses: (i) parallel evolution, (ii) long-distance dispersal and (iii) direct land connection in distant past. Himalayas and the southwestern ghats do not have more than one species common per genus; this supports Meher-Homji's hypothesis that a slow rate of migration between these regions might have led to speciation[6]. Thus different theories have been put forth to explain the geographical distribution and migration of plant species between the NE and WG montane regions. To substantiate these theories, the species that are common to both regions must be studied in more detail.

Molecular techniques provide powerful tools for studying inter and intra-specific variation and may shed light on the role of migration in the evolutionary dynamics of these plants. For example, DNA markers have been used to study phylogeography, spatial isolation and phylogenetics of many forest plant species. More specifically, the natural populations of *Ranunculus reptans* from Europe have been studied using RAPD[10]. Bartish *et al.*[11] have reported phylogenetic relationship of *Chaenomeles* from China and Japan using RAPDs and isozymes. Hagen *et al.*[12] have used RAPD and SCAR in *Cerastium arcticum* to determine trans-Atlantic dispersal and phylogeography. Sharbel *et al.*[13] have used the AFLP approach to study plants such as *Arabidopsis thaliana* for evidence of genetic isolation and postglacial colonization, while Gaudeul *et al.*[14] have examined an endangered alpine plant *Eryngium alpinum* for its genetic diversity. Genetic relationships

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among species of the genus *Diplotaxis* have been reported by Martin and Sanchez-Yèlamo[15]. Wolfe *et al.*[16] have assessed inter- and intra-species variation of genus *Penstemon* using Inter Simple Sequence Repeats (ISSRs). Hess *et al.*[17] have used ISSRs, RAPD and internal transcribed spacer-1 for phylogeographic analysis of *Olea europaea.*

E. nitida (Theaceae) is a dioecious, insect-pollinated small tree and is dispersed endozoically by birds[3]. *E. nitida* inhabits tropical and subtropical forests in cool and moist places above 1500 m and is a core member of the shola forests[3]. In India, distribution of *E. nitida* is restricted to the evergreen forests of the NE region and shola forests of the WG region (Figure 1). India's ever-increasing population has put tremendous pressure on these ecosystems. In order to increase food production, there is an increase in agricultural areas leading to reduction in the area of forests and grasslands. In the NE region a 2778 km^2 area was used for shifting cultivation from 1993 to 1997 and has caused extensive deforestation. In Meghalaya alone, 295 km^2 area has been used for a similar purpose[18]. In the WG region, smaller ranges and deforestation contribute to isolation of the shola forests. A loss of 25.6% of the total forest area has been noted in the WG region during the last 22 years, wherein the dense forest cover and open forests have decreased by 19.5 and 33.2% of their total occupation respectively, due to deforestration and land use[19]. According to Gupta[5], two factors are responsible for reduced shola cover: (i) destruction of shola forests by tree cutting and repeated fires, and (ii) reforestation with exotic plants such as *Eucalyptus*, Acacias, teak, *Cinchona* and coffee. Plant species of the shola do not regenerate in open areas even after adequate dispersal, but they do regenerate under the forest cover[6]. Sholas provide such a narrow ecological niche to their members that they are considered living fossils or relict communities, which may vanish or eventually become extinct[20].

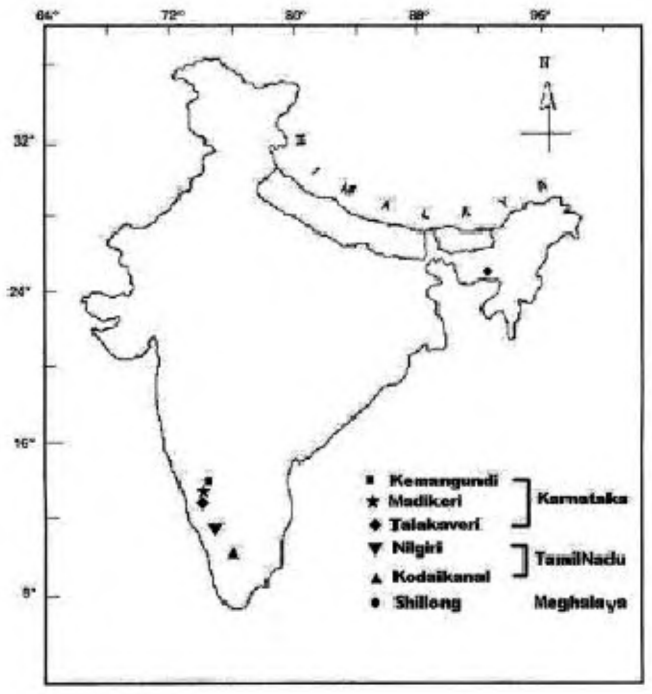

Figure 1. Map of India showing sampling locations of *E. nitida.*

To conserve this threatened forest community, it is essential to estimate the genetic diversity of its members. Being a core species of these forests, *E. nitida* has been chosen here to study the genetic consequences of the disturbance in shola forests. Previously, genetic variations in Korean population of *E. japonica*[21,22] and in Korean and Japanese populations of *E. japonica* and *E. emarginata*[23] have been studied using allozymes. However, to our knowledge, there are no reports available on the genetic structure of Indian *E. nitida* populations; hence we have made a systematic effort to estimate the genetic diversity within species and among spatially isolated populations of *E. nitida* from the NE and WG regions using the ISSR primers.

Materials and methods

Plant material and DNA extraction

Table 1 and Figure 1 give the details of sampling which was carried out at six locations in the NE Himalayan and southern WG regions. Leaf samples of individual plants at these locations were collected and stored at –40°C till further use. DNA was extracted using the modified CTAB method[24].

PCR amplification

A set of 100 ISSR primers, procured from University of British Columbia (Vancouver, Canada) were used for amplification of plant DNA. PCR reaction was carried out in 25 µl volume, as detailed by Deshpande *et al.*[25]. The amplified products were separated on 2.0% agarose gel in 0.5X TAE buffer and bands were detected by

Table 1. Collection sites of *E. nitida*

State	Location	Latitude	Longitude	No. of individuals collected
Tamil Nadu	Nilgiri	11°24′N	76°44′E	5
	Kodaikanal	10°13′N	77°32′E	5
Karnataka	Madikeri	12°26′N	75°47′E	5
	Talakaveri	12°24′N	75°31′E	8
	Kemangundi	13°18′N	75°49′E	5
Meghalaya	Shillong	25°30′N	91°52′E	9
Total				37

ethidium-bromide staining. Reproducibility of the amplification was confirmed by repeating each experiment thrice.

Data analysis

All polymorphic and reproducible bands were scored for presence (1) or absence (0), as the ISSRs are considered the dominant markers. The binary data were analysed by 'WINBOOT' software to perform bootstrap analysis and construct a dendrogram. Genetic distance was computed using WINDIST software[26]. Distance matrix was used for PCO (Principal Co-Ordinate) analysis, which was performed using the NTSYS–PC program ver. 1.8 (ref. 27).

The percentage polymorphic loci and total heterozygosity (H_T) were calculated based on the above analysis. H_T was calculated according to Nei[28] by the modified formula: $H_T = 1 - \sum pi^2$. For biallelic DNA markers, heterozygosity was calculated as $H_T = 1 - \sum(pi^2 + qi^2)$ (ref. 29), where pi is the frequency of the ith allele in the population, while qi is the frequency of the null allele for ISSR markers which are dominant in nature. Heterozygosity within populations (H_s) was also calculated using the same formula, while proportion of genetic diversity (G_{ST})[30] was further computed using formula: $G_{ST} = (H_T - H_s)/H_T$, where H_T is expected total heterozygosity and H_s is the heterozygosity within a location. The gene flow (N_m) was calculated by an indirect method to measure the total number of migrants per generation, which was estimated using a formula $N_m = 1 - F_{ST}/4F_{ST}$ (ref. 31), where standard variation F_{ST} is considered equivalent to G_{ST}[30]. AMOVA (analysis of molecular variance)[32] was used to partition the variance among the main geographic regions, among populations, within geographic regions and among individuals within the population using the program 'WINAMOVA 1.55′ provided by L. Excoffier (http: //anthropologie.unqui.ch). AMOVA–PREP [(ref. 33) (http: //www.public.asu.edu/~mmille8/amovaprp.htm)] was used for preparing files of AMOVA.

Results

Information potential of different ISSR primers in diversity analysis of E. nitida populations

Our previous study reported the usefulness of ISSR markers to analyse diversity in the populations of *E. nitida* from Nilgiri[25]. Sixteen such informative ISSR primers were, therefore, used in the present analysis. These primers mostly comprised CT and GA repeats having different mono/dinucleotide anchors and only one compound repeat with sequence $(GATA)_2$ $(GACA)_2$ (Table 2). A total of 37 individuals were screened, among which five individuals from Nilgiri as reported earlier[25] were included in the present study. This was mainly for validation of the marker system by the amplification profiles. Secondly, Nilgiri links the Karnataka and Tamil Nadu regions (Figure 1), and hence its inclusion was strategically important. This screening revealed a total of 198 bands out of which 189 bands (95.45%) were polymorphic with an average of 12.5 bands per primer in the size range of 200 to

Table 2. Comparison of primers, number of fragments scored, number of polymorphic bands, percentage polymorphism and number of unique markers for amplification profiles of 37 individuals of *E. nitida* generated using 16 ISSR markers

Primer repeat	Primer code	No. of fragments scored	No. of polymorphic bands	Per cent polymorphism	No. of unique markers
$(AG)_8T$	UBC 807	14	14	100.00	4
$(AG)_8YC$	UBC 835	17	16	94.12	1
$(CT)_8A$	UBC 814	7	7	100.00	1
$(CT)_8G$	UBC 815	9	9	100.00	1
$(CT)_8T$	UBC 813	7	7	100.00	1
$(CT)_8RA$	UBC 843	6	6	100.00	0
$(CT)_8RC$	UBC 844	11	8	72.73	0
$(CT)_8RG$	UBC 845	10	9	90.00	1
$(CA)_8RG$	UBC 848	18	18	100.00	1
$(GA)_8A$	UBC 812	12	12	100.00	1
$(GA)_8C$	UBC 811	13	12	92.31	1
$(GA)_8YT$	UBC 840	14	14	100.00	3
$(GA)_8YC$	UBC 841	17	16	94.12	0
$(GA)_8YG$	UBC 842	18	18	100.00	1
$(TC)_8G$	UBC 824	13	12	92.31	2
$(GATA)_2$ $(GACA)_2$	UBC 876	12	12	100.00	0
Total	16	198	189	–	18

Mean percentage polymorphism = 95.45.
R, purine; Y, pyrimidine.

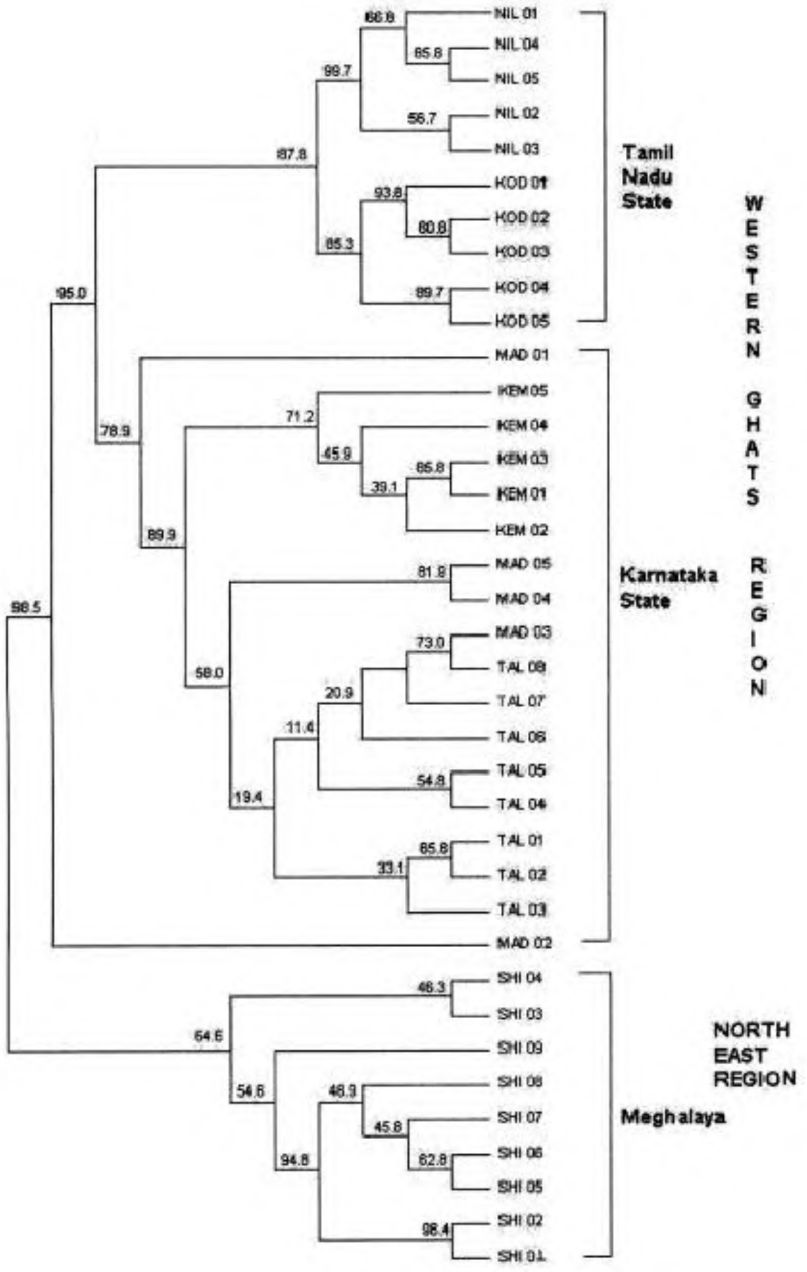

Figure 2. Phenogram produced by UPGMA analysis of 37 individuals of *E. nitida* from geographically isolated populations using ISSR markers. NIL, Nilgiri; KOD, Kodaikanal; MAD, Madikeri; KEM, Kemangundi; TAL, Talakaveri, and SHI, Shillong. Numbers at the fork indicate bootstrap values.

1400 bp. The maximum bands were produced by the repeats $(GA)_8YC$ and $(CA)_8RG$ (18 bands each), whereas minimum number of bands were produced by repeat $(CT)_8RC$ (6 bands). Interestingly, the primer with a compound repeat GATA GATA GACA GACA produced 12 bands, which were all found to be polymorphic. In all, 18 bands were unique of which maximum number (5) of bands was observed in individual Madikeri-2 and two of these ($UBC840_{1152}$ and $UBC840_{1090}$) were produced by primer $(GA)_8YT$ alone. ISSR primer $(AG)_8T$ showed maximum number of unique bands (4; Table 2).

Clustering of individuals of E. nitida based on diversity data

Based on the scoring data, a dendrogram was constructed using WINBOOT software, which showed clustering of 37 individuals in relation to their geographic location (Figure 2). Interestingly, individuals from WG and NE (Meghalaya) area formed two distinct clusters with high bootstrap value of 98.5 for the WG group, which indicates the robustness of these groups. In case of WG, individuals from Tamil Nadu formed a separate cluster with

87.8 bootstrap value and with further subgroups of Nilgiri and Kodaikanal individuals with high bootstrap values. In the case of Karnataka, Kemangundi individuals formed a separate cluster. Talakaveri individuals also remained together in one group but with interference from Madikeri individuals, wherein Madikeri-3 grouped with the Talakaveri region and the remaining two (Madikeri-4 and Madikeri-5) were close to the Talakaveri region. However, two individuals from Madikeri showed an interesting pattern. One individual from Madikeri (Madikeri-1) out-grouped from the remaining Karnataka cluster, while Madikeri-2 was out-grouped from the entire WG cluster.

Genetic differentiation of E. nitida populations

As shown in Table 3, the total hetrozygosity (H_T) among all the populations of *E. nitida* was found to be 0.2665, whereas heterozygosity for each location (H_s) ranged from 0.0864 to 0.2396, with average heterozygosity across population as 0.1345 ± 0.059. Collections from the NE region showed higher heterozygosity (H_s) of 0.2396 than those of the WG region. The degree of genetic differentiation of *E. nitida* populations (G_{ST}) ranged between 0.1009 (Shillong) and 0.6073 (Kemangundi). The gene flow calculated among all the locations considering pairwise combination at a time ranged from 0.2424 (Nilgiri and Kemangundi) to 2.6073 (Madikeri and Talakaveri; Table 4). Within Tamil Nadu, the gene flow was observed to be 0.4616 (Nilgiri and Kodaikanal), whereas it ranged from 0.5989 to 2.6073 in Karnataka. The gene flow from Shillong to other regions ranged from 0.3807 (Nilgiri) to 0.6032 (Talakaveri).

The clustering of *E. nitida* populations was corroborated using PCO analysis, which showed distinct separation between NE and WG regions (Figure 3). In the WG group too, clear separation was observed, where Nilgiri and Kodaikanal individuals (Tamil Nadu region) formed one group, while Madikeri, Talakaveri and Kemangundi (Karnataka region) formed another group. Both, dendrogram (Figure 2) and PCO analysis (Figure 3) indicated that Madikeri-1 and Madikeri-2 were separate from the Karnataka group and were situated between Tamil Nadu and Karnataka groups.

Table 3. Heterozygosity and genetic diversity within populations of *E. nitida* from different geographical locations

State	Location	H_s	G_{ST}
Tamil Nadu	Nilgiri	0.0962	0.6390
	Kodaikanal	0.1220	0.5422
Karnataka	Madikeri	0.1697	0.3632
	Talakaveri	0.0934	0.6495
	Kemangundi	0.0864	0.6757
Meghalaya	Shillong	0.2396	0.1009
Total heterozygosity (H_T)		0.2665	0.4951

In nested AMOVA analysis (Table 5), only 35.20% variation was observed among populations of *E. nitida* considering all sites together. When individuals from the regions of Tamil Nadu, Karnataka and Shillong were separately analysed on the basis of PCO and dendrogram groupings, the variation among populations was 19.47, 36.41 and 11.08% respectively. The highest variation within individuals was shown by those from Shillong (88.92%).

Discussion

Origin and migration of E. nitida

Theaceae is native to Northeast Asia, Malayan peninsula and Europe[34]. The NE region of India is a part of the Malayan floristic subcontinent, which is presumed to be one of the places of origin of angiosperms[35]. This region harbours more than one species of *Eurya*, whereas WG harbours only one species. Among the different floristic zones harbouring the same taxon, the one with higher diversity profile of that taxon is considered to be the centre of diversity[6,36]. So here we consider *Eurya* to be of Northeast Asian origin.

During the last ice age, the temperature of the Indian subcontinent was lowered by 5–7°C (ref. 6) that allowed the flora to migrate southward. Secondly, palynological reports from Chhota Nagpur[37] region and 'The Brij Hypothesis'[38] suggest the occurrence of evergreen forests in the Indian plains. During the end of the last ice age, these populations must have migrated to 'adaptive peaks', the high altitude surfaces[39] and reached the hilltops. This slow successional migration and changes in the forest communities was shown by the pollen records in Nilgiri and Kodaikanal[40].

Intra-specific molecular genetic diversity studies of *E. nitida* from NE and WG regions of India will throw light on the hypotheses of its origin and migration. Secondly, the ongoing genetic changes in these geographically isolated populations can also be revealed by these studies. In the present work an attempt has been made to achieve these goals with the help of ISSR markers.

Comparative analysis of genetic structuring of E. nitida populations from NE and WG regions of India

In our previous study on populations of *E. nitida* from Nilgiri alone, the observed polymorphism was 49.4% and heterozygosity (H_T) was 0.182 (ref. 25). In the present study, genetic diversity using the expanded set of ISSR markers revealed a high (95.48) per cent of polymorphism in the spatially isolated populations of *E. nitida* and total heterozygosity (H_T) was observed to be 0.2665, probably due to representation of numerous distant loca-

Table 4. Gene flow calculated among locations considering pairwise combination

	Nilgiri	Kodaikanal	Talakaveri	Madikeri	Kemangundi
Kodaikanal	0.4616	–	–	–	–
Talakaveri	0.4377	0.4965	–	–	–
Madikeri	0.3050	0.3938	2.6073	–	–
Kemangundi	0.2424	0.3432	0.8945	0.5989	–
Shillong	0.3807	0.4168	0.6032	0.4290	0.3990

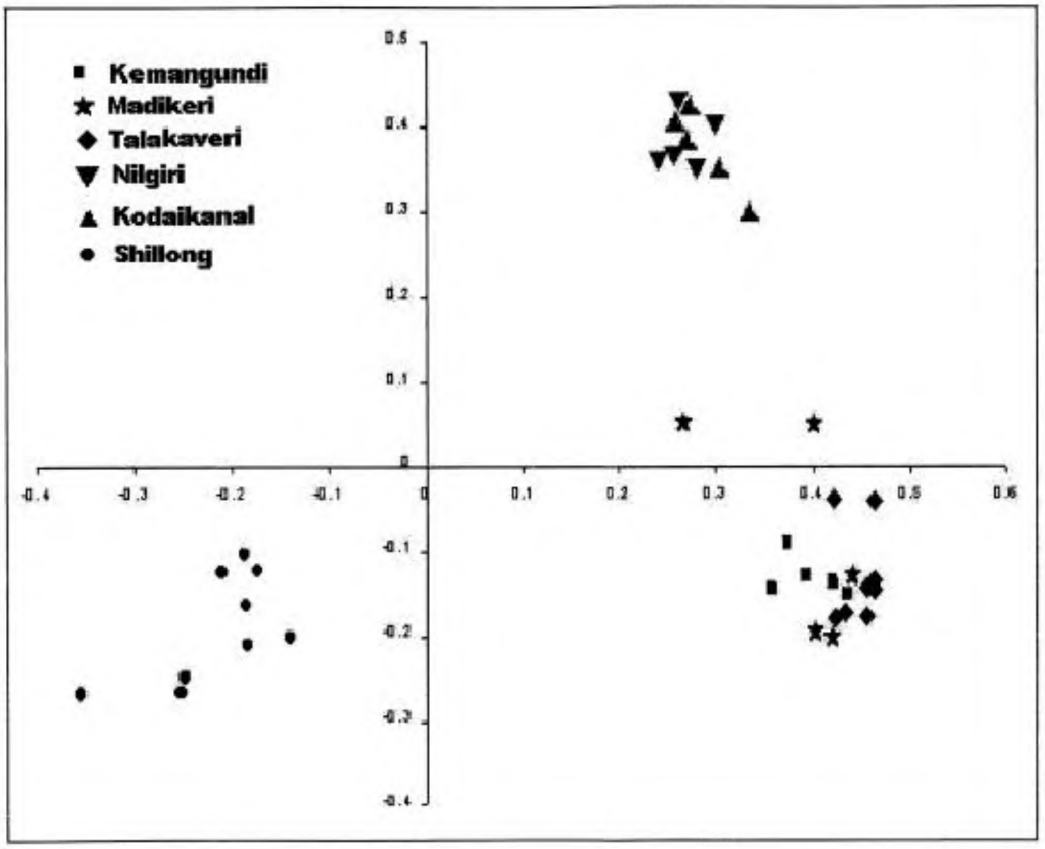

Figure 3. Representation of PCO analysis for populations of *E. nitida*.

tions. However, it is much less when compared to the other members of family Theaceae to which *E. nitida* belongs. For example, Ueno *et al.*[41] have observed 0.88 heterozygosity among the Japanese populations of *Camellia japonica* using microsatellites, and Chung and Chung[23] have reported high heterozygosity in *E. japonica* (0.324) and *E. emarginata* (0.405) among the populations of Japan and Korea respectively, using allozymes.

In our study, the average genetic diversity (G_{ST}) is high in *E. nitida* (0.4951; Table 3) than in *E. japonica* (0.072) and *E. emarginata* (0.186) as reported by Chung and Chung[23]. In the case of the WG region, Kemangundi showed the highest G_{ST}, while the lowest was observed in Madikeri (Table 3). However, these values are three times more than those in the Shillong region. AMOVA revealed higher variance (88.92%) in the Shillong population (Table 5) than in other regions, which could be because of effective seed dispersal and active pollinating agents. Some of the earlier studies have reported AMOVA value as high as 73.2% in *Cerastium articum* from the main geographic regions of the North Atlantic[12] and as low as 20.4% in the rare plant *R. reptans* from Europe using RAPD[10]. However, in the present study it is 35.20% for all the sites considered together (Table 5) and for within state analysis, they are in a range of 11.08 to 36.41%.

The gene flow calculated between two locations at a time has yielded variable values (Table 4). In Karnataka region, Talakaveri and Madikeri are about 20 km apart and exhibited the highest gene flow (2.6073). Kemangundi area is 60–70 km away from these two locations and the gene flow values for Kemangundi and these two

Table 5. Analysis of molecular variance in *E. nitida* based on 198 loci and 37 plants from six spatially isolated areas

Source of variation	Df	Actual variance component	Percentage of total variation
For all sites			
Among locations	5	10.14	35.20***
Within individuals	31	18.67	64.80***
In Tamil Nadu			
Among locations	1	4.40	19.47*
Within individuals	8	18.20	80.53*
In Karnataka			
Among locations	2	6.85	36.41
Within individuals	15	11.97	63.59***
In Meghalaya (single location)			
Among locations	1	3.53	11.08*
Within individuals	7	28.40	88.92*

Significance ***$P < 0.001$, **$P < 0.01$, *$P < 0.5$.
Df, degree of freedom.

locations ranged between 0.59 and 0.89. Thus the geographical distance between these sites is less compared to other locations, which is reflected in the higher gene flow. In Tamil Nadu, the distance between Nilgiri and Kodaikanal is more than 200 km and there was comparatively less gene flow between them (Table 4). The gene flow in Tamil Nadu and Karnataka is comparable to that within Tamil Nadu region. Shola forests are in the furrows in these regions and therefore high-altitude ridges might act as barriers, restricting the gene flow. Also, the gene flow between Shillong and other regions is comparable to those of Tamil Nadu and Karnataka, although Shillong is distinctly separated from all other locations (>2000 km; Table 4). This reveals the relative commonness or rather background genetic similarity of these fragmented plant populations. The 'indirect method' to calculate gene flow employed in the present study uses allele frequencies and also infers past gene flow[39]. On the basis of the gene flow estimates, we suggest that Talakaveri could be one of the early receivers of *E. nitida* among the surviving WG populations.

Considering comparatively low heterozygosity values along with the low ($N_m < 4$)[42] and comparable interstate gene flow values (Table 4), we can infer that shrinking of shola ecosystems is reflected in the genetic drift in *E. nitida* towards lower allelic diversity. According to Frankel *et al.*[43], such a drift is a serious threat to the genetic viability of natural populations. For a dioecious outcrossing species like *E. nitida*, such a strong drift towards homozygosity can act as a negative selection pressure. The lack of compatible and adaptive pollination and dispersal mechanism is thought to be contributing to this process.

Need for conservation strategies

Our studies revealed the low values of genetic diversity, gene flow and heterozygosity along with the strong genetic partitioning in the pocketed populations of *E. nitida*. These studies indicate the need to plan aggressive conservation strategies which should not only aim at the preservation of genotypes from one particular location, but to preserve many small populations from different locations; and for this, provision of corridors is highly recommended for these shola forest communities. Detailed studies of the genetic structures of different members of these communities along with their pollination and dispersal studies will certainly help in planning the conservation measures.

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