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RESEARCH ARTICLE

Population genetic structure of *Garcinia imberti* Bourd. an endangered endemic tree of southern Western Ghats, India

Anto M[1], Angala M[1], Jothish P S[1], Rameshkumar K B[2], Padmesh P[3] & Anilkumar C[1*]

[1]Division of Conservation Biology, [2]Phytochemistry and Phytopharmacology Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram 695 562, Kerala, India
[3]Department of Genomic Science, School of Biological Science, Central University of Kerala, Kasaragod 671 316, Kerala, India
**Email: canildeepa@yahoo.co.in*

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ABSTRACT

Assessing the genetic diversity of endemic plants is of great importance in future conservation programmes. The genetic diversity in *Garcinia imberti* from Agasthyamala Biosphere Reserve of southern Western Ghats was assessed through ISSR markers by molecular characterization with 15 primers. A total of 157 accessions from six populations were used for the study. They generated 102 amplified products, out of which 89 were polymorphic (87.25%). It produced an average of 6.8 bands per primer and 5.93% polymorphism per primer. The present study revealed that *G. imberti* has moderate level of genetic diversity at species level but differs at population level. The dendrogram constructed following UPGMA exhibited that all accessions were clustered together except Poonkulam population. The genetic diversity analysis of *G. imberti* showed that even though the populations are closely associated, every population have their own characteristic diversity and should be conserved. Among the populations, Chemunji is the largest one with more genetic diversity and may conserve as the potential source of gene pool of this species.

Introduction

The Western Ghats of India, one of the major biodiversity hotspots, harbours approximately 1275 exclusively endemic plant species of which more than 20% are tree species (1). Successful conservation strategies of such endemic plant species depend on their life history characteristics, ecological interaction with other organisms in their habitat and in part on the geographical distribution of genetic diversity (2, 3). Narrowly endemic species are susceptible to low genetic diversity and its negative consequences (4) such as inbreeding depression and loss of evolutionary potential. Populations with low genetic variation have reduced capability to adapt in the continuously changing environment and may result in reduction of population size and eventual extinction (3, 5, 6). Recent simulation model studies expected that an atmospheric temperature increase of 2-3°C over the next century would result in as many as half of the world's flora being endangered with extinction (7). Hence, knowledge on the percentage of genetic variation within and among the population of the concerned species, and the identification of populations with more genetic diversity and evolutionary potential, can provide essential information for the development of effective conservation practices. For example, an understanding on pattern of genetic diversity can help to determine which populations are genetically poor (8), whereas other populations having genetically diverse alleles and these populations may be appropriate sources for augmentation of populations (9). Accordingly, information on genetic patterns can predict the effectiveness of common management strategies, and how best to carry out those strategies. Also, recent approaches on conservation and restoration of plants may apply to preserve the genetic diversity of plant populations. This is more relevant to rare and endangered plant populations as they are usually with narrow distribution, small population sizes and geographic isolation.

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Garcinia imberti is an endemic tree restricted to the southern tip of the Western Ghats of India with endangered status as per IUCN Red List of Threatened Plants (10). This dioecious tree grows up to a height of 25 m in moist evergreen forests (Fig. 1). Male flowers borne on terminal racemose fascicles with 3-11 flowers and female flowers are usually solitary and borne on tip of branches. It was observed that, the populations are slightly female biased exhibiting more female trees to male trees (1.5:1.1). Flowering occurs from February to April and cross pollinated but fruits were also produced apomictically. *G. imberti* is found as scattered populations in evergreen forests. Previous study by Manikandan (11) recorded only about 127 adult trees of *G. imberti* in Agasthyamala hills and adjacent forest areas. Habitat loss due to expansion of tea plantations together with extensive fuel wood collection and modification of habitats are the primary threat identified for its endangered status (11). Hence, an empirical evaluation of population genetic variability is essential for a successful conservation of endangered plants (12) like *G. imberti*. The objective of the present study is to assess the percentage and distribution of genetic diversity within and amongst the population of *G. imberti* using ISSR markers, inorder to achieve the goal of preserving the species as a whole with the specific question whether there are any significant differences in genetic diversity within and among populations. Local tribal people used the bark of this tree boiled in water as analgesic and antiseptic for wounds.

Fig. 1. Habit of *Garcinia imberti* Bourd.

Materials and Methods

Sampling: Fresh young leaf samples from adult trees were randomly collected from all the six identified populations of the distributional area of *G. imberti viz.* Sankili, Cheenikkala, Ponmudi, Chemunji, Bonaccord and Poonkulam from Agasthyamala Biosphere Reserve (8°08' to 9°10' N and 76° 52' to 77° 34' E; 3500.36 km^2) of South India (Fig. 2). Among these populations, Chemunji (C) population is the larger one and continuous with more than 270 adult and immature trees, saplings and seedlings of all ages. Bonaccord (B) population is smaller one represented by about 29 adult trees and juveniles. Sankili (S) and Cheenikkala (Ch) populations are located in Shendurney Wild Life Sanctuary. Both the populations are small and have more number of adult trees (13 in Cheenikkala and 11 in Sankili) with very limited number of seedling and saplings. In all

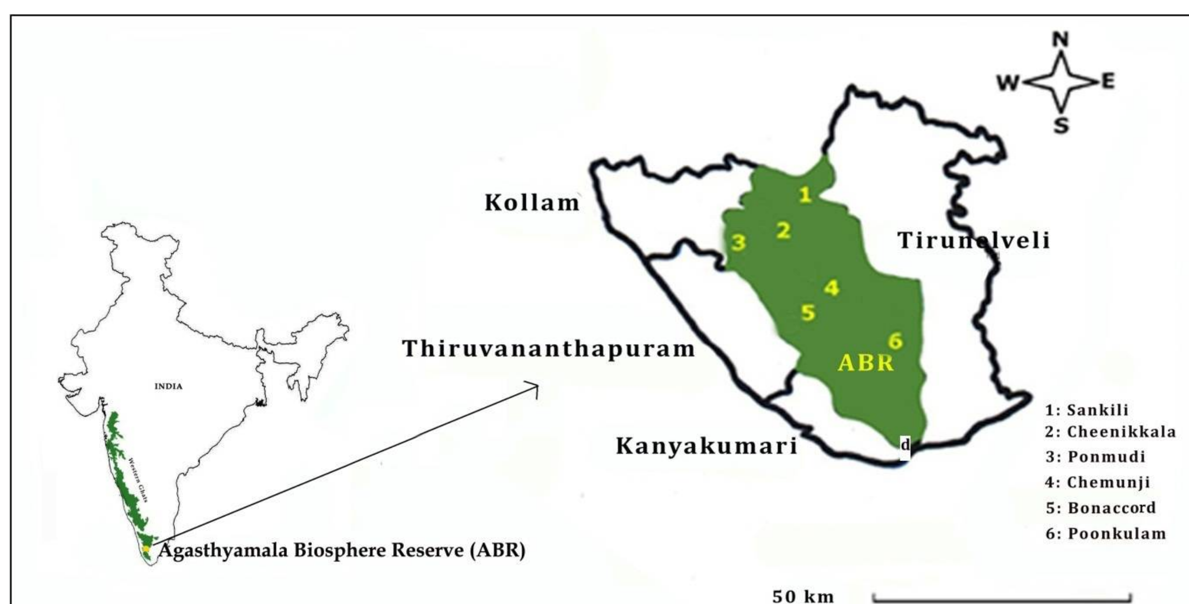

Fig. 2. Distribution map of *Garcinia imberti* populations at Agasthyamala Biosphere Reserve.

the above forests, the candidate tree is growing in association with other evergreen species such as *Agrostistachys borneensis, Cullenia exarillata, Palaquium ellipticum, Cinnamomum sulphuratum, C. chemungianum, Myristica malabarica, Litsea laevigata, Popowia beddomeana, Garcinia travancorica, G. rubro-echinata, Vateria indica* and various species of *Syzygium.* Ponmudi (P) is a hill resort of Kerala state, adjacent to Agasthyamala hills comprising a single population of *G. imberti* located in a shola forest with few adult trees and saplings. This area is highly disturbed due to frequent tourist visits. Few trees (15 adult trees) with some seedlings were also located in Poonkulam of Kalakkad Mundanthurai Tiger Reserve (Po) of Tamil Nadu state.

Sampling was performed according to the population size and accessions were collected from both adult as well as from juveniles within a population of at least 100 m distance from each other and a maximum distance of 1800 m. A total of 157 accessions were collected for genetic diversity analysis (Table 1). However, only 10 samples each could collect from Sankili, Cheenikkala, and Poonkulam populations (Table 1) as they are small as well as the individuals are so close to each other and are of same age. Leaf samples were collected and stored in an ice bucket and transferred to a -20°C deep freezer until DNA extraction (13). Herbarium specimens were prepared and deposited in TBGT (Herbarium in Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram, Kerala, India).

Table 1. Characteristics of *Garcinia imberti* populations

Populations	Sankili	Cheenikkala	Ponmudi	Chemunji	Bonaccord	Poonkulam
Latitude N	8°47'48.90"	8°47'46"	8°45'50.2"	8°41'28.0"	8°45'25"	8° 38' 20.87"
Longitude E	77°11'45.44"	77°9'2"	77°06'48.5"	77°11'04.8"	77°11'20"	77° 15'59.79 "
Altitude- (m asl)	920-1150	910-1210	900-1003	1010-1265	630-990	1050-1190
Vegetation- type	Tropical evergreen forest	Tropical evergreen forest	Tropical evergreen shola forest	Tropical evergreen forest	Tropical evergreen forest	Tropical evergreen forest
Number of samples collected	10	10	27	82	18	10
Area (km^2)	1.98	1.82	0.14	2.45	1.98	0.86
Arial distance from Chemunji population (Km)	12.8	9.3	11.1	0	4.2	10.7

Voucher specimens deposited in TBGT:

INDIA. Kerala, Thiruvananthapuram District, Chemunji, 24 April 2016, *Anto Mathew* 88401 (TBGT); Chemunji, 29 April 2016, *Anto Mathew* 88402 (TBGT); Thiruvananthapuram District, Bonaccord, 18 May 2016, *Anto Mathew* 88403 (TBGT); Bonaccord, 18 May 2016, *Anto Mathew* 88404 (TBGT); Bonaccord, 18 May 2016, *Anto Mathew* 88405 (TBGT); Bonaccord, 18 April 2016, *Anto Mathew* 88406 (TBGT); Thiruvananthapuram District, Ponmudi, 19 March 2016, *Anto Mathew* 88407 (TBGT); Ponmudi, 18 April 2016, *Anto Mathew* 88408 (TBGT); Ponmudi, 18 May 2016, *Anto Mathew* 88409 (TBGT); Bonaccord, 25 April 2016, *Anto Mathew* 88410 (TBGT); Kollam District, Sankili, 17 March 2016, *Anto Mathew* 88411 (TBGT); Sankili, 17 March 2016, *Anto Mathew* 88412 (TBGT); Sankili, 17 March 2016, *Anto Mathew* 88413 (TBGT); Sankili, 18 March 2016, *Anto Mathew* 88414 (TBGT); Kollam District, Cheenikkala, 19 March 2016, *Anto Mathew* 88415 (TBGT); Cheenikkala, 19 March 2016, *Anto Mathew* 88416 (TBGT); Thiruvananthapuram District, Attayar, 30 April 2016, *Anto Mathew* 88417 (TBGT); Thiruvananthapuram District, Pongalappara, 20 December 2019, *Anto Mathew* 88426 (TBGT); INDIA, Tamil Nadu, Tirunelveli District, Poonkulam, 18 December 2019, *Anto Mathew* 88427 (TBGT).

Genomic DNA isolation

The genomic DNA of *G. imberti* was isolated from the freeze dried leaf tissues as per the modified CTAB (Cetyl Trimethyl Ammonium Bromide) method (14). The isolated DNA samples were resuspended in 1xTE buffer of 100 µl (pH 8.0) after ethanol precipitation and kept at -20°C for future analysis. The extracted DNAs were quantified spectrophotometrically by taking the absorbance at 260 nm using Biophotometer (Eppendorf, Hamburg). The extract of DNA was diluted to 5ng/µL for PCR (polymerase chain reactions) amplification.

ISSR amplification

Fifteen random ISSR primers were selected, which produced clear and reproducible fragments and were used for the final analysis (Table 2). The PCR reaction mixture was prepared to 25 µl volume containing 50 ng of DNA template, 0.2 mM dNTP mix, 1X reaction buffer, 20 pmol primer and 1 unit Taq DNA Polymerase (Finnzymes, India) and double distilled water. The reaction mixture concentration and PCR conditions were standardized by trials. The amplification was performed using Eppendorf Thermocycler with a hot start at 94° C for 2 min; followed by denaturing at 94°C for 15 sec of 35 cycles each; annealing for 15 sec at 37°C; and product extension time for 5 min at 72°C. The amplified products were resolved in agarose gel (1.5%) containing Ethidium Bromide in a submarine electrophoresis unit (BioRad Inc.) and visualized under Gel documentation system (15).

Data Analysis

All the readable amplified fragments from the gels were scored based on the fragment presence or absence and denoted as '1' or '0' respectively. The binary matrix was analyzed using POPGENE program version 1.31, (16) for different genetic diversity parameters like Nei's genetic distance, Nei's gene diversity at population level (h), Shannon's diversity index (I), expected number of alleles (Na), and coefficient of genetic differentiation (G_{st}). The cluster analysis was performed using the Nei's genetic

Table 2. Primers and the number of bands produced by them used for genetic diversity analysis

ISSR Primers	Sequence	Maximum no. of bands produced/ population	Maximum no. of polymorphic bands produced/population
808	AGAGAGAGAGAGAGAGC	9	8
823	TCTCTCTCTCTCTCTCC	6	6
824	TCTCTCTCTCTCTCTCG	6	6
826	ACACACACACACACACC	4	4
827	ACACACACACACACACG	6	6
829	TGTGTGTGTGTGTGTGC	5	5
834	AGAGAGAGAGAGAGAGYT	8	6
835	AGAGAGAGAGAGAGAGYC	9	7
836	AGAGAGAGAGAGAGAGYA	7	6
840	GAGAGAGAGAGAGAGAYT	9	6
841	GAGAGAGAGAGAGAGAYC	7	7
844	CTCTCTCTCTCTCTCTRC	8	7
845	CTCTCTCTCTCTCTCTRG	7	5
847	CACACACACACACACARC	5	5
848	CACACACACACACACARG	6	5
	Total	**102**	**89 (87.25%)**
	Bands/primer	**6.80**	**5.93**

distance (dissimilarity) matrices and corresponding phenograms produced for the 157 samples using the unweighted pair group method with arithmetic averages (UPGMA) and dendrogram was constructed using MEGA software version 6. The gene flow (N_m) among the populations was calculated on the assumption that the members in the populations are at random mating and be in Hardy-Weinberg equilibrium and populations follow island model described by Wright (17). Level of gene flow (N_m) was estimated following Wright's F statistics, F_{ST} = 1/4 (N_m+1) from which Nm was calculated as $N_m = (1 - F_{ST})/4F_{ST}$. The G_{st} value obtained from the POPGENE analysis was substituted for F_{ST} value and derived the gene flow level (18).

Results

A total of 102 amplified products were generated by the 15 primers (Table 2), of which 89 were polymorphic (87.25%) with a mean of 6.8 bands for each primer and 5.9% polymorphism for each primer. However, different populations exhibited different percentage of polymorphic bands such as Sankili with 84.1%, Cheenikkala with 86.2% Ponmudi with 74.1%, Chemunji with 92.7%, Bonaccord with 65.0% and Poonkulam with 92.6%. The number of products produced by the primers ranged from 4 to 9; primers 808, 835 and 840 produced maximum number of amplicons (9) and primer 826 produced minimum amplicons (4) (Table 2). At species level, *G. imberti* showed moderate level of genetic diversity with a mean value of 0.41 ± 0.1 and ranged among different populations from 0.21 to 0.41 and Shannon's diversity index of 0.59 ± 0.1 (Table 3). The observed heterozygosity (Ho) was also high and the observed number of alleles was 2 with 100% polymorphism at species level. However, the mean value of observed heterozygosity (Ho) in the different samples of *G. imberti* was 0.41 ± 0.00 and the mean value of the average expected heterozygosity (He) was 0.18 ± 0.00. The heterozygosity values and degree of genetic differentiation (G_{st}) is shown in Table 3. The diversity indices of individual populations showed that, the effective number of alleles (ne) and observed number of alleles (na) across the populations were found to be 1.71 ± 0.2 and 2.0 ± 0.0, respectively (Table 3).

Population genetic differentiation and gene flow level

Genetic differentiation amid the populations studied (G_{st}, assuming Hardy-Weinberg Equilibrium) is 0.55, showing that the only 45% variation was found within populations (Table 3). The Nm (gene flow) values between accessions were calculated on the assumption that the study samples follows the inland model of Wright (17) which expect a connection between the number of migrants an accession receives per generation and *Fst*. The gene flow level estimated as 0.41 individuals per generation among populations, telling that exchange of genes between *G. imberti* populations was comparatively low. The individuals within Chemunji population showed high heterozygosity (Ho) and low genetic differentiation (G_{st}). Ponmudi population showed low rates of gene flow, high genetic differentiation and low genetic diversity.

The dendrogram constructed following UPGMA showed that accessions are clustered into two major groups and six subgroups and Poonkulam is totally distinct from other populations (Fig. 3). The dendrogram showed that accessions are clustered geographically as east (Poonkulam) and west (rest of the five populations) of the Western Ghats (Fig. 3). Among the subgroups, Sankili and Cheenikkala are distant than other populations. All accessions from Chemunji population come under the same clade of the dendrogram showed genetically alike accessions. Accessions from Ponmudi populations showed two separate clades, and showed proximity to Sankili – Cheenikkala populations.

Discussion

Population genetic structure defined as the group of individuals which share a common gene pool, determines the capacity to be enhanced or altered by selection (19). The present study showed that *G.*

Table 3. Different genetic diversity indices assayed in *Garcinia imberti*

	Ho	He	Gst	Nm	na	ne	h	I
At species level	0.41 ± 0.0	0.18 ± 0.0	0.55	0.4	2.00 ± 0.0	1.71 ± 0.2	0.41 ± 0.1	0.59 ± 0.1
Individual populations (Mean ± SD)								
S	0.39 ± 0.0	0.28 ± 0.0	0.29	1.22	1.93 ± 0.3	1.69 ± 0.3	0.39 ± 0.1	0.57 ± 0.2
Ch	0.39 ± 0.0	0.23 ± 0.1	0.41	0.71	2.00 ± 0.0	1.68 ± 0.3	0.39 ± 0.1	0.57 ± 0.1
P	0.21 ± 0.0	0.14 ± 0.0	0.55	0.40	1.80 ± 0.4	1.33 ± 0.3	0.21 ± 0.2	0.34 ± 0.2
C	0.41 ± 0.0	0.18 ± 0.0	0.36	0.88	2.00 ± 0.0	1.75 ± 0.2	0.42 ± 0.1	0.60 ± 0.1
B	0.32 ± 0.0	0.23 ± 0.0	0.30	1.17	1.93 ± 0.3	1.57 ± 0.4	0.32 ± 0.2	0.48 ± 0.2
Poo	0.32 ± 0.0	0.19 ± 0.0	0.38	0.83	1.80 ± 0.4	1.57 ± 0.4	0.32 ± 0.2	0.46 ± 0.3

(S- Sankili; Ch- Cheenikkala; P- Ponmudi; C- Chemunji; B- Bonaccord and Poo- Poonkulam; na- observed number of alleles; ne- effective number of alleles; h- Nei's (1973) gene diversity; I- Shannon's diversity index; Ho- observed heterozygosity; He- expected heterozygosity; G_{st}- genetic differentiation and Nm- gene flow)

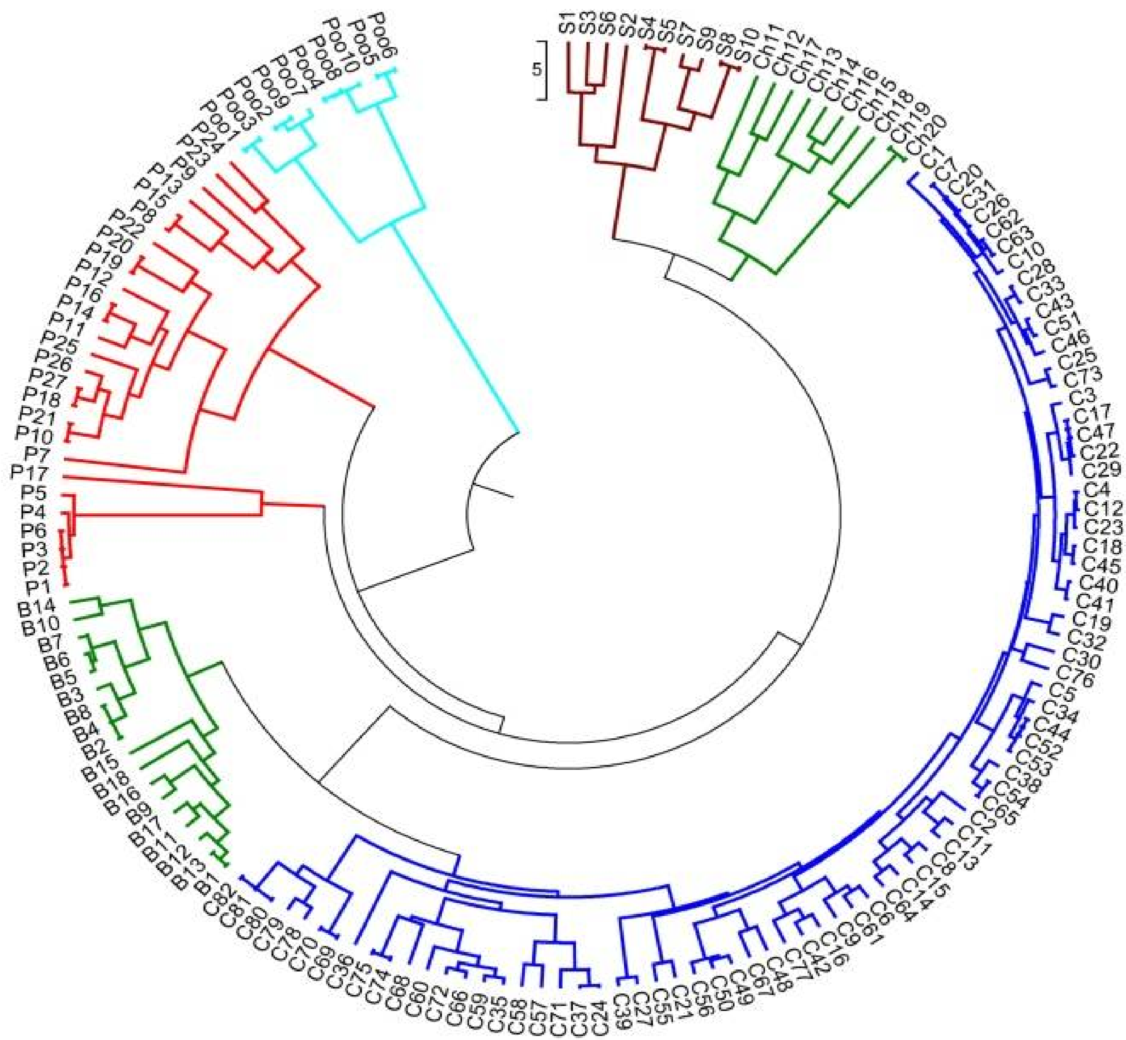

Fig. 3. Dendrogram of genetic diversity analysis on *Garcinia imberti*
(S- Sankili; Ch- Cheenikkala; P- Ponmudi; C- Chemunji; B- Bonaccord; Poo- Poonkulam).

imberti has moderate level of genetic diversity. Genetic diversity is one of the fundamental evolutionary variables that associate with size and viability of population and the persistence of species. As an endemic tree species, the expected heterozygosity (Ho) of *G. imberti* within populations was high. Knowledge of genetic diversity within and between populations offers essential information for the development of appropriate management strategies focused towards their protection (20). In *G. indica*, another endemic of the Western Ghats, exhibited low levels of genetic diversity and was attributed to apomixis as most plants are derived not by cross pollination (21). Similar type of observations were reported in *G. atroviridis*, endemic to Peninsular Malaysia (22). However, the high level of genetic variation in *G. cambogia* observed the accessions from geographically distinct Kerala regions could be due to outcrossing by insects, which causes the higher gene flow rate within and among the populations (23).

Different parameters of genetic diversity such as percentage of polymorphism, Nei's genetic diversity, Shannon's diversity index and average heterozygosity were moderately high in the present

study. This results reveals that *G. imberti* have moderate genetic diversity similar to some other endemics such as *Poeciloneuron pauciflorum* (24), *Agave victoriae-reginae* (25), *Antirrhinum charidemi* and *A. valentinum* (26). The Shannon's diversity index varies from 0 to 1, and high genetic diversity is indicated by values closer to 1 (27). It was reported that the degree of polymorphic locus is not an applicable measure of the genetic variation and thus, the heterozygosity (genetic diversity) is more important (28). Reports are on the genetic diversity between the accessions of *Varronia curassavica* obtained Shannon's diversity index *i.e.,* 0.42, polymorphism *i.e.,* 97.98%, expected heterozygosity of 0.27 using 14 primers (29). Reports are also on the plant species with separate sex, the overall within population genetic diversity was moderate since biased sex ratios may harmfully affect the genetic diversity due to higher genetic drift (30). This may also applicable to the dioecious *G. imberti* populations with slightly female biased sex ratio (personal observation).

Maintenance of genetic variability of an endangered plant species is one of the major aims for conserving that species (31). The analysis of population genetic structure and reproductive capacity of *G. imberti* have imperative implications for conservation strategies. The distribution of genetic diversity plays a significant role for conserving species composition (32, 33). There are studies revealing that determining the highest conservation value of a population is not that simple (34). The present study showed that Chemunji population with high allele richness and genetic diversity indicated the occurrence of cross breeding and comparatively more gene flow than other populations. Allelic richness is a direct measure of genetic diversity that target at selecting the populations for conservational importance (35, 36). The maximum G_{st} value signified the level of genetic differentiation among the populations (37).

The *G. imberti* samples from different forest ranges of Agasthyamala Biosphere Reserve showed the clustering according to the spatial arrangement of the populations (Fig. 3). The dendrogram showed individual accessions belonging to different populations clustered distinctly indicated the genotypes were more closely allied among themselves than other populations. Except Poonkulam samples, all other samples are from western portion of the Ghats. The clustering indicated that gene flow occurred mainly within the populations rather than between populations. The geographical distance of populations restricts gene flow between populations which resulted in reduced genetic variability. According to Nybom (38), pollen and seed dispersal, geographic distribution range, successional stages and mating systems are some of the factors that can decide the percentage and distribution of genetic variability amid and within populations. Sankili and Cheenikkala are adjacent populations but more distant than other populations. An interesting observation is that, the geographical location of Ponmudi population. It is an isolated population found in a shola forest separated by large hillocks. Also, they have very less rate of gene flow and high genetic differentiation than other studied populations and genetic diversity but showed two sub-clusters indicated that they are of different origins or affected by genetic drift. In a study it was revealed that small populations of *Pulsatilla patans* exhibited the signs of genetic loss due to genetic drift (34). The decline of genetic diversity within populations at marginal geographical area may limit their evolutionary capability delaying adaptation to environmental factors (39-41). Isolated populations may adaptively diverge and showed reduced gene flow rate (42). Low rate of genetic diversity could lessen the plant strength and limit a population's capacity to react to varied eco-physiological influences through selection and adaptation (43).

Partitioning of genetic variation in *G. imberti* (G_{st} = 0.55) was high with limited historical gene flow across a wide geographic range. The reduced genetic diversity between populations may be due to geographic separation and reduced gene flow (Nm = <1) between the uneven populations (44). Nm value of 0.41 showed that, gene flow rate between populations is limited. Since most of the populations are separated by long distance (>8 km), and limited gene flow (both pollen and through seeds) within and among populations are the main factor shaping the distinct genetic structure of *G. imberti*. In the long-term viewpoint, reduce in genetic variation leads to lower population adaptability and enhanced the risk of extinction under varied habitats (34). The *G. imberti* populations are suffered with small population size, reduced gene flow due to lack of pollinators and seed dispersers (11, 45) wide geographic separation among the populations besides various stress and slow seedling growth (46) invite serious attention to conserve the species.

Conclusion

Designing conservation strategies for endangered species require a good knowledge about the level and distribution of genetic diversity. Discontinuous distribution and small population size along with high allele richness invite serious attention to conserve the species. *G. imberti* populations are declining due to various stochastic effects along with slow seedling growth. If such pressure continues without sustainable and appropriate management of the genetic resources the diversity and natural abundance of the species will be declined. Complementary *ex- situ* conservation, propagation and cultivation methods need to be undertaken for the protection and maintenance of the existing genetic diversity of *G. imberti*. The findings can serve as a guide to preserve every similar population or species from endangerment.

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Authors' contributions

AM and AM collected the leaf samples from the field, carried out the work, analysed the data. AM, PPP, JPS, RKB and AC interpreted the data and helped in manuscript writing.

Conflict of interests

The authors declared that they have no conflict of interest.

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