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Genetic diversity in *Oroxylum indicum* (L.) Vent., a threatened medicinal plants from India by ISSR analysis

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Oroxylum indicum is an important threatened traditional medicinal plant native to Indian subcontinent. Inter Simple Sequence Repeats (ISSR) markers were used to assess the genetic diversity and population genetic structure of 39 accessions of *O. indicum* belonging to South and North East India. A total of 92 discernible bands were generated from 17 primers, with 81 (88.04%) being polymorphic, indicating high genetic diversity at the species level. The Nei's gene diversity (*h*) was estimated to be 0.2526 within populations, and 0.4027 at the species level. Analysis of molecular variance (AMOVA) showed that the genetic variation was found mainly among populations (68%), but variance within populations was only 32%. In addition, Nei's differentiation coefficients (G_{ST}) was found to be high (0.3733), confirming the relatively high level of genetic differentiation among populations. The unweighted pair group method of arithmetic-average (UPGMA) clustering also demonstrated high genetic differentiation between South and North East Indian populations. The estimated gene flow (Nm) from G_{ST} was 0.8393. It indicated that the fragmentation of populations and anthropogenic activities might result in specific evolutionary history. Based on the genetic data, a conservation strategy is proposed for this threatened species.

Keywords: Genetic diversity, genetic structure, ISSR, *Oroxylum indicum*

Introduction

Oroxylum indicum (L.) Vent, (Bignoniaceae) commonly called "midnight horror tree", is a bat-pollinated, small deciduous tree species native to the Indo-Malayan region and occurs in many parts of India. It is an important ingredient of ayurvedic and indigenous medical preparations for over thousands of years[1]. Roots are used in common ayurvedic preparation called "Dasamularist", considered to be an astringent, anti-inflammatory, antihelminthic, antibronchitic, antileucodermatic, antirheumatic, antianorexic and for treatment of leprosy[2]. It is also used in other Ayurvedic formulations such as Amritarista, Dantyadyarista, Narayana Taila, Dhanawantara Ghrita, Brahma Rasayana, Chyavanaprasa Awalwha etc[3]. Stem and root bark contains flavones, namely oroxylin A, chrysin, and scutellarin-7-rutinoside, traces of alkaloid, tannic acid, sitosterol and galactose[4]. Seed extract exhibits antimicrobial, analgesic, antitussive and anti-inflammatory properties[5].

O. indicum is scattered in the moist deciduous forests in peninsular India. It blooms at night from April to August, and is propagated naturally by seeds. The fruit is about 80 cm in length, hanging down from the branches and fruit set is extremely poor due to poor pollination efficiency. The fruit capsule dehisces at the edges and the flat papery thin seeds (500-700 per pod) are dispersed by wind. Due to indiscriminate harvest and habitat degradation, its natural population is on the verge of extinction. Conservation Assessment and Management Plan, the process developed by Conservation Breeding Specialist Group (CBSG) of International Union of Conservation of Nature (IUCN), has identified this species as highly threatened. It has been Red listed in some States of the Indian Union, following IUCN criteria and categories. It has been categorized as endangered in Kerala, Maharashtra, Madhya Pradesh and Chathisgarh and vulnerable in Karnataka and Andhra Pradesh Assam, Karnataka, Meghalaya, and Sikkim[6,7]. The percentage of decline of populations recorded in Karnataka is 20, Kerala 50, Andhra Pradesh 30, Maharashtra 50, Meghalaya 30 and 50 in Assam, Sikkim[8] The understanding of population genetic structure is important for formulating an effective conservation strategies for this species. Although much efforts have been directed for its biochemical characterization and *in vitro* propagation[7,9], genetic diversity of *O. indicum* is poorly characterized[10].

Assessment of the distribution level of genetic diversity within a plant species contributes vital

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information on regarding its evolutionary history and is critical to the development of effective conservation and management practice[11-14]. Several aspects of conservation biology, such as loss of genetic diversity and restoration of threatened populations, can only be addressed by detailed population genetic studies[15]. Among various molecular marker tools, inter-simple sequence repeats (ISSR) based on PCR amplification, have been widely used for population genetic studies of various plant species, including several medicinal plants[11,12,16,17]. ISSR amplifies inter-microsatellite sequences at multiple loci throughout the genome without previous knowledge of DNA sequences and its primers are designed from microsatellite region[16]. Furthermore, they are universal, quick, easy to apply, highly reproducible, polymorphous, cost efficient and requires lesser amounts of DNA (25-50 ng)[16]. This is not always the case with the other popular DNA markers such as RAPD, RFLP and AFLP[16,18,19]. The present study elucidate the genetic diversity and structure of *O. indicum* populations in South and North-East India using ISSR markers with an objective to provide insight to facilitate conservation management.

Materials and Methods

Exploration and Collection

An extensive survey was conducted to study the natural distribution of *O. indicum* populations in South and North-East India. A total of 39 accessions were collected from different locations along with their geographical coordinates. The study region in South India covered mainly Karnataka state and its border region of Kerala with a total of twenty accessions whereas North-East India covers Assam state and its border region of Meghalaya with nineteen accessions. The altitudes of the collection sites were ranged from 3 meter (Mogral, Kerala) to 984 meter (Umiam, Meghalaya). DIVA GIS[20], a software commonly used for mapping and analyzing biological distribution data, was used to create geographical distribution map for *O. indicum*. The input data used in DIVA GIS was given in Table 1. The map was obtained from the above software representing the distribution of *O. indicum* in various regions of South and North East India (Fig. 1).

DNA Extraction and ISSR-PCR Analysis

Genomic DNA was extracted from fresh leaves (0.5 g) of *O. indicum* using CTAB method[21] with modifications as described previously[22]. Quantity and quality of the DNA samples were estimated by comparing band intensities on a 0.8% agarose gel and using a spectrophotometer (Shimadzu UV-1800). ISSR-PCR reaction was performed in a volume of 10 µl containing 40 ng template DNA, 0.5mM dNTPs (Chromous Biotech, Bangalore, India), 0.15U *Taq* DNA polymerase (Chromous Biotech), 0.5µM ISSR primers from University of British Columbia (The Michael Smith Laboratories, University of British Columbia, primer set # 9, Vancouver, BC, Canada) and 1X PCR buffer (10 mM Tris HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$) (Merck). 17 reproducible primers were selected from 100 ISSR primers for the present study based on their consistent amplification profiles

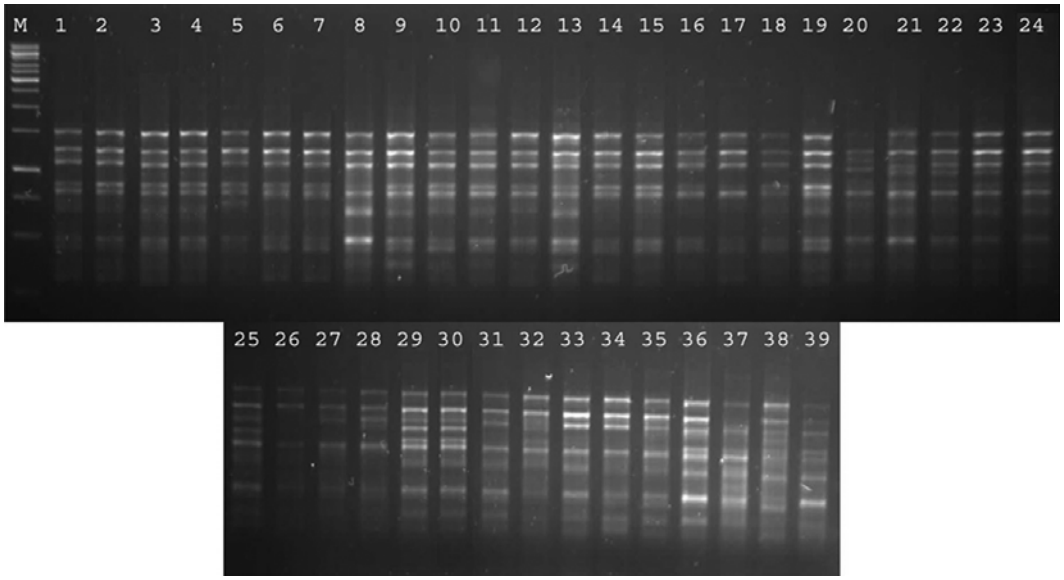

Fig 1 — ISSR fingerprint of 39 accessions of *O. indicum* using primer UBC 873. Lane M, 1Kb marker.

(Table 2). A control PCR tube containing all components but no genomic DNA was run with each primer to check any contamination. The reactions were carried out in a DNA thermocycler (Eppendorf mastercycler gradient, Germany). The thermocycler was programmed for an initial denaturation step of 94ºC for 4 min, followed by 34 cycles at 94ºC for 1 min, 45 sec at the specific annealing temperature of each primer (45 to 58°C) and 72ºC for 1 min and a final extension at 72ºC for 8 min and a hold temperature of 4ºC at the end. After amplification, the reaction products were subjected to electrophoresis in 1.5% agarose gels in 1X TAE buffer stained with 5µg ml^{-1} ethidium bromide and photographed under UV light with the help of a gel documentation system (Syngene). 1 kb molecular ladder was used as marker to know the size of the fragments. All the PCR results were tested for reproducibility at least three times. Bands that did not show fidelity were eliminated.

Table 1 — Details of *O. indicum* accessions used for genetic diversity analysis

Sl. No.	Accession no.	Place of collection	No of plants per population
	South Indian Population		
1	KERRET-11	Mogral, Kerala	6
2	KERRET-12	Kutar, Kerala	1
3	KERRET-13	Pazhappa, Kerala	7
4	KARRET-118	Mookambika Wild Life Sanctuary, Karnataka	3
5	KARRET-120	Jadkal , Karnataka	6
6	KARRET-124	Kalmanja, Karnataka	1
7	KARRET-125	Peramogru, Karnataka	1
8	KARRET-127	Perne, Karnataka	1
9	KARRET-128	Puthoor Thaluk , Karnataka	1
10	KARRET-131	Pallodi, Karnataka	1
11	KARRET-132	Narikkumbu, Karnataka	1
12	KARRET-133	Mappiladakka, Karnataka	3
13	KARRET-134	Mani, Karnataka	6
14	KARRET-135	Narahari Nagar, Karnataka	1
15	KARRET-136	Parkala, Karnataka	1
16	KARRET-138	Shivapura, Karnataka	1
17	KARRET-139	Someshwara Wild Life Sanctuary, Karnataka	1
18	KARRET-141	Seethanadi, Karnataka	2
19	KARRET-142	Seethanadi, Karnataka	1
20	KARRET-143	Agumbe ghat, Karnataka	5
	North East Indian Population		
21	ASRET-1	Thalni, Assam	3
22	ASRET-2	Khamar, Assam	3
23	ASRET-3	Amgury , Assam	1
24	ASRET-4	Bherakushi , Assam	2
25	ASRET-7	Dimu, Assam	2
26	ASRET-9	Modertoli Forest Gate, Assam	3
27	ASRET-17	Forest Range Office, Diphu, Assam	1
28	ASRET-19	Deopani, Assam	3
29	ASRET-20	Juriadolong, Assam	4
30	ASRET-23	Kaziranga National Park Central Zone, Assam	20
31	ASRET-24	Kuruahabari, Assam	1
32	ASRET-26	Mazline, Assam	2
33	ASRET-27	Bagori, Assam	1
34	ASRET-28	Rangolu, Hatimura, Assam	2
35	ASRET-29	Gotunga , Assam	1
36	ASRET-30	Kolangpur, Assam	2
37	ASRET-31	Khethri , Assam	1
38	MLRET-4	NBPGR, Umiam Meghalaya	4
39	MLRET-5	Umdihar, Meghalaya	6

Table 2—ISSR primers used for PCR amplification of *O. indicum* and total number of amplified fragments generated from 39 accessions*

Sl. No.	Primer No	Sequence (5'→ 3')	Total No. bands	No. polymorphic bands	% of polymorphic bands
1	818	$(CA)_8G$	5	5	100
2	822	$(AC)_8G$	6	6	100
3	825	$(AC)_8T$	7	6	85.7
4	827	$(TC)_8A$	5	5	100
5	834	$(AG)_8YT$	5	5	100
6	844	$(CT)_8RC$	5	4	80
7	848	$(CA)_8RG$	3	2	66.6
8	855	$(AC)_8YT$	7	6	85.7
9	857	$(AC)_8YG$	8	7	87.5
10	864	$(ATG)_6$	5	4	80
11	866	$(CTC)_6$	4	4	100
12	873	$(GACA)_4$	6	5	83.3
13	874	$(CCCT)_4$	6	5	83.3
14	888	BDB $(CA)_7$	3	2	66.6
15	889	DBD $(AC)_7$	7	5	71.4
16	902	CTC $(GT)_8$	4	4	100
17	906	$(AC)_8YA$	6	6	100
Total			92	81	88.04

*B = (C, G, T); D = (A, G, T); R = (A, G); Y = (C, T).

Data Analysis

Amplified products, which were reproducible and consistent in performance, were scored for band presence (1) or absence (0) and a binary qualitative data matrix was constructed. Percentage of Polymorphic Bands (PPB) was calculated by dividing the number of polymorphic bands by the total number of bands surveyed. The binary matrix was used to determine the genetic diversity, genetic differentiation and gene flow using the software PopGene 32 version 1.31[23]. Genetic diversity within and among populations were measured by PPB, effective number of alleles (ne), observed number of alleles (na), Nei's[24] gene diversity (*h*) and Shannon's information index (*I*). At the species wide level, total genetic diversity (H_T) and genetic diversity within populations (H_S) were calculated. To estimate the genetic divergence among populations, we also calculated the relative magnitude of genetic differentiation among populations ($G_{ST} = (H_T - H_S)/H_T$). Corresponding estimates of gene flow (Nm), *i.e.* the average per generation number of migrants exchanged among populations, was estimated using the formula: $Nm = 0.5 (1 - G_{ST})/G_{ST}$ [25].

In addition, an analysis of molecular variance (AMOVA)[26] was performed to calculate the partitioning of genetic variance among and within population using GenAlEx ver. 6.41[27]. The permutation number for significance testing was set to 999 for all the analysis. To explore the genetic relationships among all populations, an unweighted pair group method of Arithmetic average (UPGMA) dendrogram was constructed based on the matrix of Nei's genetic distance by the program TFPGA, version 1.3[28]. Given that the above estimation of allele frequencies from dominant markers requires the assumption of Hardy–Weinberg equilibrium.

Results

Genetic Diversity

Using the 17 ISSR UBC primers that showed the best resolution in the amplification profiles, 92 clearly identifiable bands were obtained from 39 accessions of *O. indicum* populations from South and North-East India. Of these, 81 (88.04%) bands were polymorphic and the remaining 11 (11.96%) were monomorphic (Table 2). At the population level, 59.78% and 61.96% PPB were observed in South and North-East Indian populations respectively. An average of 5.41 bands per primer were yielded and the sizes of bands were ranged from 450 bp to 1900 bp (Fig 2). 100% polymorphic bands were recorded in seven primers. Since ISSR markers are dominant, each band represents the phenotype at a single bi-allelic locus. The ISSR primers identified in this study will be used for further genetic analysis of *O. indicum*.

Although both populations studied exhibited similar level of genetic diversity, North-East Indian population revealed a little higher Nei's genetic diversity (*h*) (0.2624 ±0.051) and Shannon information index (*I*) (0.3772 ±0.071) than South Indian population (h = 0.2427 ± 0.087, I = 0.3525 ± 0.0684) (Table 3). The average values of *h* and *I* were estimated as 0.2526 ± 0.006 and 0.3772 at the population level and 0.4027 ± 0.025 and 0.571 ± 0.035 at the species level respectively. The observed number of alleles (*na*) and effective number of alleles (*ne*) across the populations were found to be 1.8804 ± 0.052 and 1.7567 ± 0.05, respectively.

Population Genetic Differentiation

Genetic differentiation among the populations studied (G_{ST}, assuming Hardy-Weinberg Equilibrium) is 0.3733, showing that the majority of variation (62.67%) was distributed within populations, similar to results of AMOVA analysis (Φ_{ST} = 0.323) (Table 3). The overall level of inferred gene flow (Nm) was estimated as 0.8393 individuals per generation among populations, suggesting that exchange of genes between populations was slow. Nei's genetic distance

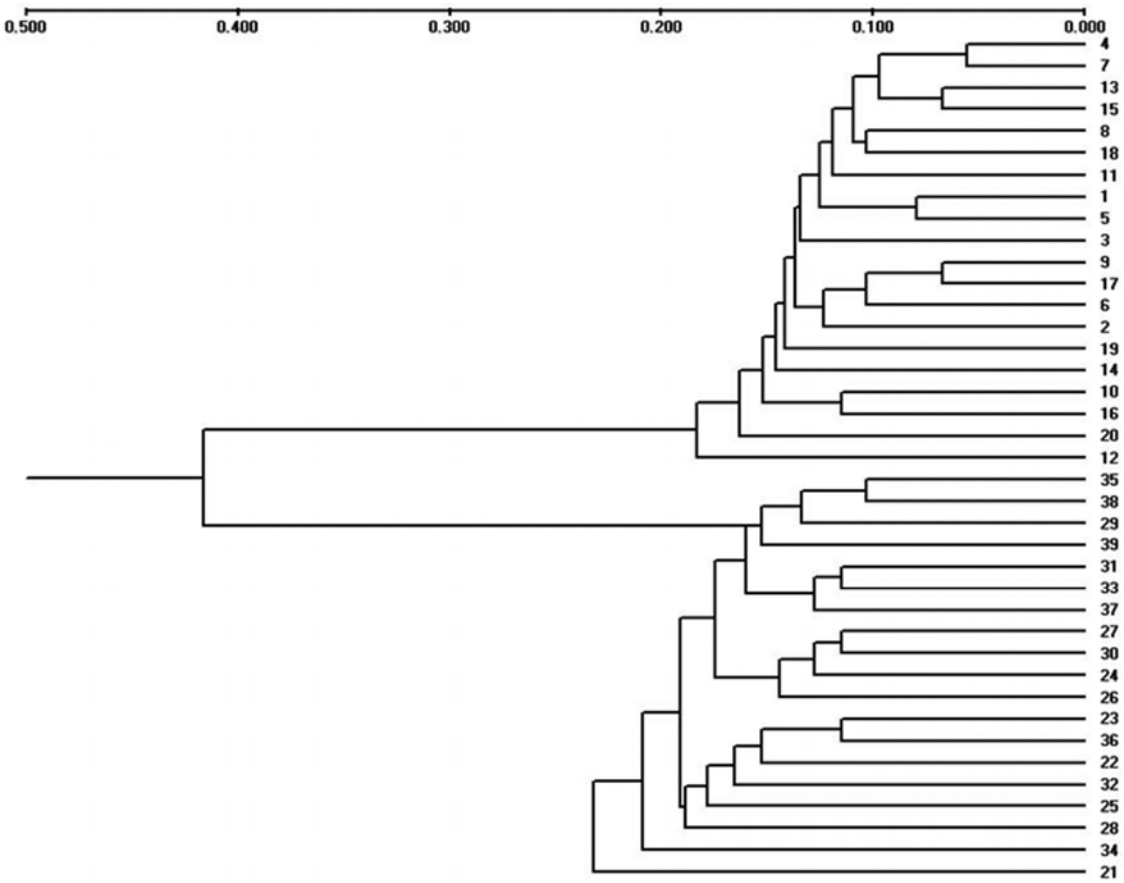

Fig 2 — UPGMA dendrogram illustrating the genetic relationships among 39 accessions of *O. indicum* belonging to South and North-east India, based on Nei's (1978) unbiased genetic diversity.

Table 3 — Analysis of genetic diversity for South and North East Indian populations of *O. indicum* detected by ISSR.

Population	*H*	*I*	PPB	G_{ST}	*N*m	AMOVA (Φ_{ST})
South Indian	0.2427 ±0.087,	0.3525 ±0.0684	59.78%			
North East Indian	0.2624 ±0.051	0.3772 ±0.071	61.96%			
Mean	0.2526 ±0.006	0.3772	60.87%			
Species level	0.4027 ±0.025	0.571 ±0.035	88.04%	0.3733	0.8393	0.323

h, Nei's (1973) diversity index; *I*, Shannon's information index; PPB, percentage of polymorphic loci; G_{ST}, genetic differentiation between populations (Nei's); *N*m, estimated gene flow; Φ_{ST}, genetic differentiation between populations estimated by AMOVA analysis.

(D) between the populations was 0.5151. The UPGMA tree based on Nei's[24] genetic distance (D) was shown in Fig. 3. It indicates the relationship between all 39 accessions belonging to South and North-East Indian populations. It was interesting to note that all accessions in these two regions separately clustered which reveals the existence of two geographically distinct populations.

Discussion

Genetic Diversity

The aim of the present study was to estimate the extent of genetic variation in *O. indicum*. Till date, the efforts to unravel the genetic diversity in the species *O. indicum* is negligible. Prior to this work, only a report on RAPD analysis of the population from Andhra Pradesh state in India is available[10]. It is proposed that high genetic diversity has a positive effect on the long-term persistence of species by increasing their ability to adapt to changing environmental conditions[29]. Accordingly, less genetic variation would affect population viability by reducing individual fitness[30]. Tropical trees are reported to have high levels of intra-population genetic diversity. Similarly the present study revealed a relatively high level of genetic diversity among the two populations (South and North East) of *O. indicum* based on ISSR markers (Table 2). It was also observed that the diversity within the populations was moderate. The mean genetic

Fig. 3 — Geographical distribution map of accessions of *O. indicum* takne for the study

diversity within population (H_S = 0.2526) was similar to the average value of the outcrossing species (H_S = 0.27) and higher than that of the short-lived perennial plants (H_S = 0.20)[31]. It is generally believed that threatened plant species tend to maintain a low genetic diversity than common species[32]. However, some threatened species show high levels of genetic variation even within extremely narrow distributions[33]. In a particular population, the number of alleles at a locus and their frequency of distribution can be evaluated as polymorphism and heterozygosity is the probability that two alleles taken at random from a population can be distinguished by the given marker technique[34].

Pollen and seed dispersal, successional stages, geographic distribution range, and mating systems are some of the factors that can determine the levels and distribution of genetic variability among and within populations[31]. *O. indicum* is a perennial, outcrossing, self-incompatible species and its breeding nature has been well documented[35,36]. It is pollinated by bat and two bat species,

Rousettus leschenaultia (Desmarest) and *Cynopterus sphinx* Vahl have been suggested as the possible pollinators of *O. indicum*[37,38]. *C. sphinx* is the main pollinator in India and it is proposed that dependence on the single bat pollinator in different regions is probably detrimental to the species in different parts of the tropics causing decreased fecundity and decline in population size[36]. Natural fruit-set in *O. indicum* is poor due to pollen limitation and insufficient xenogamous pollination which limits widespread distribution of this species. In addition, poor seed germination and seedling establishment in natural habitat also contribute to the threatened status of the species. Interestingly, the seeds recorded 99% germination and establishment in our green house conditions which indicates that lack of proper germination conditions in natural habitat is the major set ball for its reduced sapling establishment. The breeding behaviour of this species greatly influences its low level genetic variability within populations compared to other tree species. The observed high genetic diversity among population is thought to be due to the geographical distance between the populations studied. The inter as well as intra-specific variation in this species might have resulted because of sexual recombination, segregation, together with mutations, acted on by natural selection. Breeding system, genetic drift or genetic isolation of populations can cause high level of genetic differentiation among plant species populations[39]. Similarly, scattered distribution of a species and topographical barriers can lead to difficulties in pollen and seed dispersal, consequently to limited gene flow among populations[40]. Narrow distributional range, high habitat specificity, low natural regeneration are some of the highlight of *O. indicum* populations studied.

Population size greatly influences genetic variation in plant species. We observed in field survey that the population number and size in *O. indicum* are rapidly decreasing in wild due to over exploitation and other anthropogenic activities. As an important ingredient in Ayurvedic formulations, *O. indicum* has been harvested continuously for hundreds of years. In addition, *O. indicum* is a softwood tree and hence it is highly fragile to natural calamities which may also contribute to its threatened status. The above mentioned threats to the natural populations of *O. indicum may* resulted in loss of genetic variation.

Population Genetic Differentiation

The genetic structure of plant populations reflects the interactions of various factors including the long-term evolutionary history of the species (shifts in distribution, habitat fragmentation and population isolation), genetic drift, mating system, gene flow and selection[41]. The genetic structure of *O. indicum* obtained in the present study indicated that the among-population differentiation coefficients (G_{ST} = 0.3733) were higher than the average coefficients of short-lived perennial species (G_{ST} = 0.32, Φ_{ST} = 0.41) and outcrossing plant species (G_{ST} = 0.22, Φ_{ST} = 0.27)[30]. The geographical separation and discontinuous distribution in populations of *O. indicum* contribute to the high level of population differentiation and on the other side, the outcrossing nature of this species counteract the ultra high population differentiation. The effective gene flow among the populations (Nm = 0.8393) was lower than one successful migrant per generation which greatly pressurizes rapid genetic differentiation. Cluster analysis revealed that all the accessions from a particular population clustered together which indicated that gene flow in the studied *O. indicum* populations occurred mainly within the populations rather than between populations. The ripened fruits of *O. indicum* dehisces along their sutures and the thin papery seeds fly away to long distances which might have enhanced the gene flow at some extant. The current gene flow and extremely reduced distribution of this plant, often with less than 5 individuals per population (Table 1), can be interpreted as the species was once widespread in the past and later it was fragmented by habitat degradation and exploitation in medical preparations.

Implication for Conservation

Successful management and conservation of populations of threatened species depend on a good understanding of the distribution of genetic variation in the species[42]. The primary objective of conservation is to preserve the evolutionary potential of species by maintaining as much genetic diversity as possible. The population genetic structure and reproductive capacity have significant implications for conservation strategies. The low level of reproductive capacity may inhibit the multiplication of populations, which will indirectly cause the loss of genetic diversity of this species.

Though *O. indicum* maintains relatively high genetic diversity among the populations as revealed in this study, discontinuous distribution and extremely small population size along with high genetic differentiation invite urgent and serious attention to conserve the species. Even though the species is important in human health care and has been used for

centuries, it is neither domesticated nor conserved efficiently. From the light of current study, we suggest integrated strategy to conserve the species *in situ* as well as *ex situ*. Since single or even a few plants will not represent the whole genetic variability in *O. indicum*, there appears a need to maintain sufficiently large populations *in situ* to conserve genetic diversity in *O. indicum* and avoid genetic erosion. As an important traditional medicinal plant, promoting domestication and cultivation are necessary for satisfying market demand and protecting the wild resource. The species has short juvenile period and fast reproductive stages. We have successfully established different accessions of the plant from different parts of country in our field gene bank which strongly indicates it as a suitable species for domestication. Complementary *ex situ* conservation, propagation and cultivation methods need to be urgently undertaken for protection and maintenance of genetic diversity in these rapidly declining genetic resources of *O. indicum.* The parents should be propagated vegetatively and trees from different populations in one regions should be put together to form a 'seed orchard'. Seed orchards for North East and South India to be established separately for continuous monitoring of the natural populations, habitat management and will help in conservation of this species. In conclusion the geographical separation and discontinuous distribution in populations of *O. indicum* contribute to the high level of population differentiation and presumably contribute out crossing nature of this species the high population differentiation.

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