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Assessment of Genetic diversity of *Gymnema sylvestre* (Retz.) R.Br. from Western Ghats and Eastern India, India

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Abstract

To device management strategies for conservation of plant, the complete knowledge of genetic variation within and among populations of plant species is essential. Inter simple sequence repeat and Random amplified polymorphic DNA markers were used to study the genetic diversity and population genetic structure in 117 accessions of 11 populations of *Gymnema sylvestre*, an important anti diabetic plant from Western Ghats and Eastern India of India. A total of 7 ISSR and 6 RAPD selected primers produced 3474 and 4104 discernible bands respectively, with 100% polymorphism. The Nei's gene diversity (*h*) was found to be 0.27 and 0.26 at the species level based on ISSR and RAPD markers respectively indicating high genetic diversity. The Shannon's index (*I*) was estimated to be 0.42 and 0.41 at the species level based on ISSR and RAPD markers respectively. The analysis of molecular variance showed that the genetic variation was found equally within populations (ISSR = 55% and RAPD = 51%) and among populations (ISSR = 45% and RAPD = 49%) both. In addition, Nei's differentiation coefficient (G_{ST}) was found to be high (ISSR = 0.70 and RAPD = 0.60) and the gene flow (*Nm*) was low (ISSR = 0.21 and RAPD = 0.33), confirming the high population genetic differentiation. There was high genetic differentiation and the low gene flow in this species of plant, which suggests that conservation should emphasis on preserving the populations and which will also help the concerned authorities in designing strategies on the conservation of *G. sylvestre*.

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Introduction

Gymnema sylvestre R.Br. (Retz.) is a member of the milkweed family (Asclepiadaceae). It is a vigorous climbing perennial vine whose leaves have been used for centuries in the traditional Indian system of medicine (Komlavalli *et al.*, 2000). The leaves of the plant have the property to inhibit the taste of sweetness in the mouth when chewed.

G.sylvetre is widely grown in India, Malaysia, Sri Lanka, Australia, Indonesia, Japan, Vietnam, Tropical Africa and SW China (Saneja *et al.*, 2010). In India the plant is native to tropical and subtropical regions and specially distributed wild in the forests of Central India, Western Ghats, Konkan, Tamilnadu, Karnataka, Goa and Madhya Pradesh (Komlavalli *et al.*, 2000).

Despite its extensive use in diabetes and other conditions, little is known about the distribution of genetic variation across their geographical ranges. Data related to genetic diversity within and between populations are essential for formulating appropriate management strategies for the conservation of plant species. Several studies have been carried out on genetic diversity in India. The study on 18 samples of *G.sylvestre* from Kerala using 15 RAPD primers has revealed high polymorphism (Nair *et al.*, 2006). Similarly polymorphism on 11 progenies of *G. sylvestre* from Uttar Pradesh using 40 RAPD primers have been reported (Osman *et al.,* 2013). In Maharashtra genetic diversity was carried out on 22 accessions of *G. sylvestre* using ISSR and RAPD markers that resulted high level of gene differentiation (Shahnawaz *et al.*, 2011). High polymorphism is reported on 5 plant samples from Haryana using ISSR marker (Khatak *et al.*, 2014). It is interesting to note that all the above authors have obtained high genetic diversity within the populations of *G. Sylvestre*. However, available genetic diversity reports explored few locations, small samples size, RAPD fingerprinting, no elaborate data on population diversity e.g. gene frequency, Shannon's information index.

Diversity exists at three levels: Genetic diversity, Species diversity and Ecosystem diversity (Shiva *et al.*, 1994; Rao *et al.,* 2002). There is an assumption that the conservation of ecosystems and habitats also conserves species, and that the conservation of species also conserves genetic diversity. But the conservation of species does not necessarily conserve the genetic diversity within species. So, in nature conservation strategies, the conservation of genetic resources has not been done practically (Swedish Environmental Protection Agency, 2008). The conservation of biological diversity should emphasise on preventing the disappearance of genetically distinct populations rather than the sole prevention of the extinction of species. This will also lessen the risk of extinction, even in a longer time perspective as the ability of a population to adapt to the environmental changes depends on genetic variability or diversity of the population (Avise *et al.*, 1996; Ellstrand *et al.*, 1993).

The objective of the present study was to carry out genetic diversity analysis of 117 accessions of *G.sylvestre* collected from 11 diverse agro climatic locations of two distinct ecological regions of India, namely Western Ghats (WG) and Eastern India (EI) using ISSR and RAPD molecular markers.

Materials and methods

Survey and collection of plant material

A total of 117 accessions were collected during the study which includes 101 samples from Karnataka, Maharashtra and Goa in the region of WG and 16 samples from West Bengal in the region of Eastern India (Table 1). The collection of plants was done under the supervision of taxonomists and the distance between each population was maintained to be 10 kms. For all samples latitude, longitude and altitude was recorded using global positioning system enabled device (Garmin GPSMAP 60 CSx). The species was authenticated at Regional Medical Research Centre (ICMR), Belagavi and voucher specimens were deposited (Reference number: RMRC-584, RMRC-585, and RMRC-586).

DNA isolation

Genomic DNA of *G. sylvestre* was isolated from leaves using CTAB method (Doyle *et al*., 1987) with some modifications. Purified DNA was quantified spectrophotometrically using nano-drop spectrophotometer (JH Biosciences) at 260 nm. Quality of DNA was determined by agarose gel (1%) electrophoresis against standard lambda (k) phage DNA using Bio-Rad mini horizontal submarine unit (Bio-Rad Inc. USA).

PCR amplifications

Out of 20 ISSR primers, we selected 7 primers which resulted into clear, sharp and maximum bands (Table 2). The PCR amplification was carried out in a thermal cycler (Bio-Rad, USA) with an initial temperature of 94°C for 3 min, 40 cycles of denaturation at 94°C for 45 sec, annealing at 37°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min.

Out of 10 RAPD primers, we selected 6 primers which resulted into clear, sharp and maximum bands (Table 2). The PCR amplification was carried out in a thermal cycler (Bio-Rad, USA) with an initial temperature of 94°C for 5 min, 45 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 45 sec, extension at 72°C for 2 min and final extension at 72°C for 5 min.

The amplified products were electrophoresed on 1% (w/v) agarose gels, in 1X TAE Buffer at 80 V for 3-4 hours and then stained with gel red and documented using gel-documentation system (Syngene, UK).

Data analysis

Amplification with each primer was repeated three times and amplified products, which were reproducible and consistent, were scored for presence (1) or absence (0) of each band. The bands were considered as rare, shared and similar on the basis of their amplification less than 15%, up to 70% and above 70% accessions respectively (Grativol *et al*., 2011). The resulting binary data matrix was analyzed using softwares.

Assuming Hardy Weinberg equilibrium, statistical package POPGENE 1.32 (Yeh *et al*., 1997) was used to calculate genetic diversity within and among populations in terms of (Shannon's information index), h (Nei's genetic diversity) (Nei, 1973), H_T (total heterozygosity), H_S (heterozygosity within population), G_{ST} (degree of population differentiation), N_m (number of migrants per generation or gene flow) (Slatkin *et al*., 1989). GenAlex ver. 6.4 (Peakall *et al.,* 2006) was used to generate principal co-ordinate analysis (PCoA) and analysis of molecular variance (AMOVA) to calculate variation among and within populations.

The genetic distance matrix was also used to compare the populations based on Nei's genetic distance (Nei, 1972), by making an unweighted pair group method with arithmetic mean (UPGMA) dendogram after bootstrapping 1000 times using the TFPGA (Tools For Population Genetic Analysis) software (Miller, 1997).

The measure of variability at specific loci, polymorphic information content (PIC) was calculated using the formula $1\text{-}p^2\text{-}q^2$, where p is the frequency of present bands and q is frequency of no bands (Ghislain *et al*., 1999).

Results

ISSR and RAPD profiling

The seven selected ISSR primers produced 3474 reproducible bands with a mean of 496.29 bands per primer and all (100%) were polymorphic. The number of amplified bands ranged from 382 (UBC-814) to 615 (UBC-826) having a range of 200 to 3500 bp fragment size. The total number of loci generated by all seven primers was 133 with an average of 19 per primer. The mean value of PIC for primers was 0.282 which ranged from 0.232 (UBC 814) to 0.354 (UBC 826). Out of total bands generated by ISSR markers 45.86% were rare, 52.63% were shared and 1.51% was similar bands (Table 2).

The six selected RAPD primers produced 4104 reproducible bands with a mean of 684 bands per primer, all of which (100%) were also polymorphic. The various RAPD primers amplified the number of bands from 640 (RPI02) to 745 (RPI03) having a range of 300 to 3500 bp fragment size. The total number of loci generated by all six primers was 128 with an average of 21.33 per primer.

Table 1. Sampling details of *G. sylvestre* populations.

Population Sites	Regions	Population codes	No. of individual	Latitude	Longitude	Elevation (MSL in meter)
West Midnapore	EI	WMP	05	E 22.27154	N 86.53867	072
Darjeeling	EI	DLG	09	E 26.47431	N 88.21542	163
Kolkata	EI	KLK	02	E 22.550611	N 88.33786	020
Belagavi	WG	BGM	10	E 15.68209	N 74.50470	694
Shimoga	WG	SMG	07	E 14.18634	N 74.90784	592
Mysore	WG	MYS	02	E 12.27962	N 76.66541	825
Uttar Canada	WG	SRS	33	E 14.58555	N 74.79646	583
Sindhudurg	WG	SDG	06	E 15.96201	N 23.99564	762
Goa	WG	GOA	12	E 15.30924	N 73.94579	088
Tilari	WG	TLR	11	E 15.26282	N 74.61993	790
Kolhapur	WG	KLP	20	E 16.82231	N 74.12827	951

EI: Eastern India, WG: Western Ghats, MSL: Mean sea level.

The mean value of PIC for primers was 0.315 which ranged from 0.281 (RPI04) to 0.351 (RPI06). Out of total bands generated by RAPD markers 38.23% were rare, 58.59% were shared and 3.13% were similar bands (Table 2).

Table 2. ISSR and RAPD primers used in this study with respective polymorphism and PIC value.

Primer	Primer sequence 5' – 3'	Total no. of Bands (TNB)	No. of monomorphic bands (NMB)	No. of polymorphic bands(NPB)	Percentage of polymorphism (%)	PIC Value
			ISSR (UBC set no # 9)			
UBC 814	CTCTCTCTCTCTCTCTA	382	0	382	100	0.232
UBC 815	CTCTCTCTCTCTCTCTG	523	0	523	100	0.270
UBC 826	ACACACACACACACACC	615	0	615	100	0.354
UBC 841	GAGAGAGAGAGAGAGAYC	499	0	499	100	0.269
UBC 855	ACACACACACACACACYT	518	0	518	100	0.299
UBC 876	GATAGATAGACAGACA	448	0	448	100	0.268
UBC 880	GGAGAGGAGAGGAGA	489	0	489	100	0.298
Total	-	3474	0	3474	100	0.282
			RAPD (RPI)			
RPI 01	AAAGCTGCGC	683	0	683	100	0.330
RPI02	AACGCGTCGC	640	0	640	100	0.285
RPI03	AAGCGACCTC	745	0	745	100	0.337
RPI04	AATCGCGCTG	655	0	655	100	0.281
RPI05	AATCGGGCTG	659	0	659	100	0.310
RPI06	ACACACGCTG	722	0	722	100	0.351
Total	-	4104	0	4104	100	0.315

UBC: University of British Columbia, RPI: Reading Primer International, PIC: Polymorphic Information Index.

Genetic polymorphism

At the species level, the percentages of polymorphic bands (PPBs) based on ISSR and RAPD fingerprinting was found to be 97.08% and 98.46% respectively but when seen according to the geographical area it was found to be 58.49% and 100% for EI and WG respectively based on ISSR markers. However based on RAPD markers it was found to be 83.05% and 100% for EI and WG respectively.

Genetic relationships

All 133 and 128 polymorphic loci obtained from ISSR and RAPD primers were used to estimate the genetic divergence among accessions by calculating the Nei's genetic distance (Nei, 1972). The values of genetic distance (GD) ranged from 0.01 to 0.34 (ISSR) and 0.01 to 0.33 (RAPD).

All accessions were grouped into four main clusters based on UPGMA dendogram. All the populations were clustered according to their geographical area except the Uttara Kannada populations which were clustered with Kolhapur (ISSR marker) (Fig.1).
Similarly principle coordinate analysis generated 4 (ISSR) and 5 (RAPD) clusters.

All the populations were grouped according to their geographical area except Kolhapur population which was found to be clustered with Belagavi, Shimoga and Mysore. Uttara Kannada populations were clustered separately into two clusters (Fig. 2).

Partitioning of genetic variation

The total genetic variation was partitioned into variation among population and within population (*P = 0.001*). AMOVA for ISSR matrix demonstrated 55% genetic variation among populations and 45% within populations (Table 3).

Table 3. Analysis of Molecular variance.

Marker	Source	df	SS	MS	EV	%	P Value
ISSR	Among Population	10	1216.303	121.630	11.301	55%	0.001
	Within Population	106	978.843	9.234	9.234	45%	0.001
	Total	116	2195.145		20.536	100%	0.001
RAPD	Among Population	10	1222.610	122.261	11.219	51%	0.001
	Within Population	106	1133.013	10.689	10.689	49%	0.001
	Total	116	2355.624		21.907	100%	0.001

%: total variance (percentage of total variance contributed by each component), *Df*: degrees of freedom, *SS:* sum of squares, *MS:* mean square, *EV:* variance component.

The coefficient of gene differentiation (G_{ST} = 0.70) calculated using POPGENE showed large variation at species level whereas at population level it was found to be 0.27 & 0.63 for EI and WG respectively. Gene flow (N_m) was less than 1 (0.21) at species level whereas at population level it was found to be 1.35 & 0.29 for EI and WG respectively (Table 4).

AMOVA for RAPD matrix demonstrated 51% genetic variation among populations and 49% within populations (Table 3). The coefficient of gene differentiation (G_{ST} = 0.60) calculated using POPGENE showed large variation at species level whereas at population level it was found to be 0.27 & 0.53 for EI and WG respectively. Gene flow (N_m) was less than 1 (0.33) at species level whereas at population level it was found to be 1.37 & 0.44 for EI and WG respectively (Table 4).

Genetic diversity

The populations showed relatively high level of genetic diversity h = 0.27 and I = 0.42 using ISSR marker at species level whereas at population level it was found to low h = 0.17 & 0.29 and I = 0.26 & 0.44 for EI and WG respectively. Similarly the heterozygosity among population (H_T) was 0.2660 while within population (H_S) it was found to be 0.0779 (Table 4).

Similarly high level of genetic diversity h = 0.26 and I = 0.41 was observed using RAPD marker at species level whereas at population level it was found to be h = 0.20 & 0.31 and I = 0.33 & 0.48 for EI and WG respectively. Heterozygosity among population (H_T) was 0.2612 while within population (H_S) it was found to be 0.1041 (Table 4).

Table 4. Various genetic diversity parameters analysed in *G.sylvestre* based on Nei, 1987.

Population codes		na	ne	*h*	*I*	*Ht*	*Hs*	G_{ST}	N_m	PPB%
ISSR	EI	1.5849	1.2703	0.1677	0.2618	0.1546	0.1127	0.2708	1.3462	58.49
	WG	2.0000	1.4781	0.2863	0.4393	02662	0.0996	0.6257	0.2991	100
	Species	1.9708	1.4573	0.2740	0.4212	0.2660	0.0791	0.7027	0.2116	97.08
RAPD	EI	1.8305	1.3036	0.2019	0.3286	0.2116	0.1550	0.2676	1.3686	83.05
	WG	2.0000	1.5223	0.3139	0.4769	0.2936	0.1369	0.5336	0.4371	100
	Species	1.9846	1.4284	0.2636	0.4096	0.2612	0.1041	0.6014	0.3314	98.46

na: observed number of alleles, ne: expected number of alleles, *h*: Nei's gene diversity at population level, *I*: Shannon's index of genetic diversity, *Ht*: heterozygosity at the polymorphic loci, *Hs*: average heterozygosity, *GST*: degree of genetic differentiation, *Nm* gene flow.

Discussion

G. sylvestre has very high medicinal value which makes it one of the highly marketed plants. It is therefore a very important plant species from the medicinal and economical perspective. The genetic structure of plant populations is a result of the interactions of various processes such as long term evolutionary history of the species (shifts in distribution, habitat fragmentation and population isolation etc.), gene flow, genetic drift, mutation, and natural selection (Schaal *et al.*, 1998).

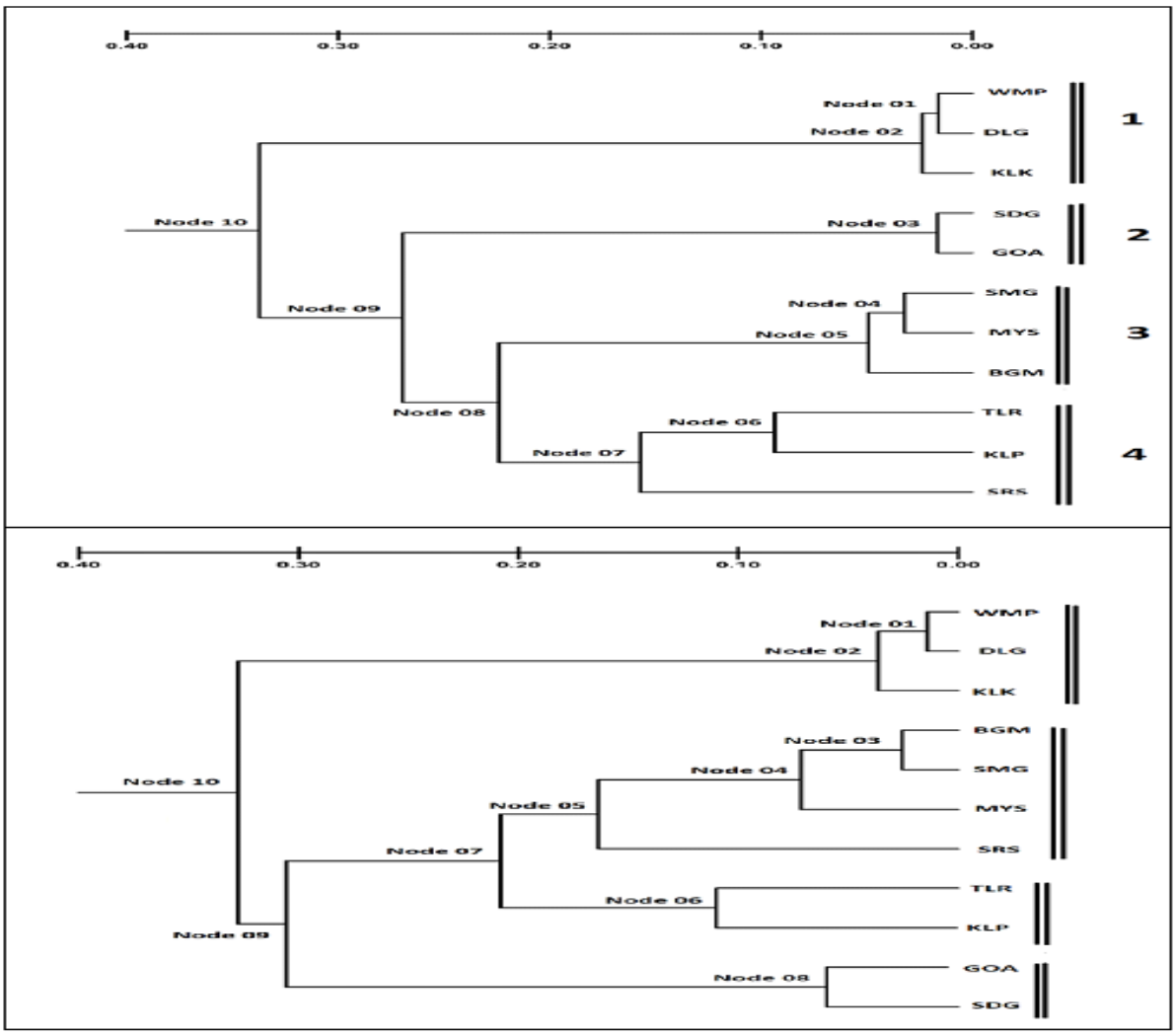

Fig. 1. UPGMA dendrogram based on Nei's genetic distance between populations of *G. sylvestre* using ISSR and RAPD primers.

Genetic diversity studies are independent of sample size even sample of two individuals could also yield heterozygosity estimate if a sufficient number of loci are generated (Gorman *et al.*, 1979). For the present study healthy numbers of 117 accessions of *G.sylvestre* were collected from different regions of EI and WG.

In the present study, ISSR and RAPD markers were used to analyse the genetic diversity and population structure of *G. sylvestre*. Efficiency of the markers was studied by evaluating rare, shared and similar bands (Bhagwat *et al.*, 2014). From these data we could identify particular accession (rare bands) and the resemblance among the accessions (shared and similar bands) from different geographical locations (Tatikonda *et al.*, 2009; Gravitol *et al.*, 2011; Bhagwat *et al.*, 2014). UBC815 amplified the maximum number of rare bands and had high SPI (6.20), which makes it the most suitable primer to identify particular accession as well as for detection of polymorphism in *G. sylvestre* populations. Similarly in RAPD primers, RPI 02 and RPI 04 amplified the maximum number of rare bands. However RPI03 had high SPI (7.75), which makes RPI 02 and 04 as the most suitable primers to identify particular accession and RPI03 as the primer of choice for use in detection of polymorphism in *G. sylvestre* populations.

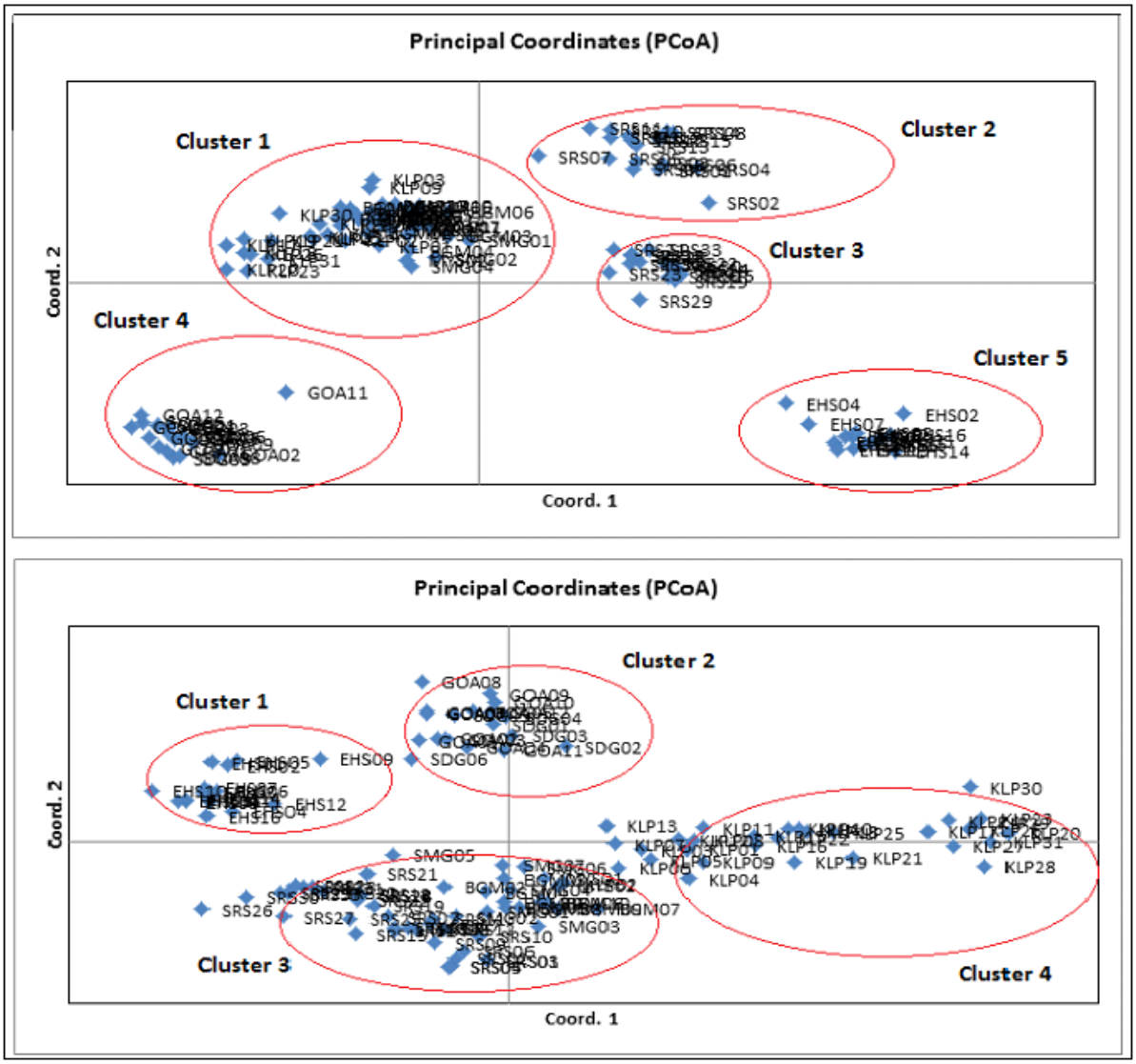

Fig. 2. Principal Coordinate analysis among 117 accessions from 11 populations of *G. sylvestre* for ISSR and RAPD marker.

The polymorphic information content (PIC) and the polymorphism rate were taken as one of the measure to analyse the genetic diversity in *G.sylvestre*. The mean value of PIC was found to be 0.315 (RAPD) and 0.282 (ISSR) whereas percentage of polymorphism was 100% for both the markers, indicating high level of polymorphism. PIC value describes the level of polymorphism where PIC > 0.5, 0.5 > PIC > 0.25 and PIC < 0.25 shows high, medium and low polymorphism respectively (Vaiman *et al*., 1994; Xei *et al*., 2010).

In this study, AMOVA revealed that genetic variability among the populations (ISSR = 55% and RAPD = 51%) is higher than that within the populations (ISSR = 45% and RAPD = 49%) in *G.sylvestre*. The threshold value of G_{ST}> 0.25 indicates remarkable population differentiation (Han *et al*., 2007). In our study the G_{ST} value was found to be 0.70 (ISSR) and 0.60 (RAPD) at species level indicating high genetic differentiation. This might be due to the large geographical distance between EI and WG or genetic drift and inbreeding depression which leads to increase in small and isolated populations (Templeton *et al.,* 1990; Tomimatsu *et al*., 2003; Zhao *et al*., 2009). Though *G. sylvestre* is widely distributed, it had fragmented habitats and sizes of isolated populations were small which might be responsible for high genetic differentiation among the populations. However, it was 0.27 (ISSR) and 0.63 (RAPD) in EI populations and 0.27 (ISSR) and 0.53 (RAPD) in WG populations. A study on *G.sylvestre* on 22 accessions from Maharashtra region showed lower differentiation than our populations [G_{ST} = 0.35(RAPD); G_{ST} = 0.41(ISSR)] (Shahnawaz *et al*., 2011).

It is known that improvement in adaptation to changes with environment is better when there is more genetic variation which will improve gene flow also (Buckley *et al*., 2010; Kremer *et al*., 2012). Low gene flow (N_m< 1) results into consequential local differentiation which leads to genetic drift instead higher gene flow (N_m> 1) makes the population fit for survival (Statkin, 1987).

Therefore 1 to 10 migrants per generation are essential for restoration and resistance of genetic drift and preventing natural selection (Lopez *et al.,* 2009; Blanquart *et al*., 2011). In *G.sylvestre*, the gene flow at species level was quite low, 0.21 (ISSR) and 0.33 (RAPD). It is quite low to effectively prevent differentiation by genetic drift. This could be due to very large distance between EI and WG which might have played as a barrier for gene flow. However, within population it was 1.35 (ISSR) and 1.37 (RAPD) in EI populations and 0.29 (ISSR) and 0.43 (RAPD) in WG populations which is quite high. Low gene flow in WG could be due to habitat fragmentation, low seed dispersal and geographic isolation. Geographic isolation limits the amount of gene flow via both pollen and seeds (Pfeifer and Jetschke, 2006). Populations were separated by geographic barriers (e.g., mountains, rivers) and anthropogenic facilities, which has affected gene flow via seed and pollen dispersal among populations. Similar study carried out with 22 accessions of *G.sylvestre* from Maharashtra region showed higher gene flow among populations [N_m = 0.92 (RAPD); N_m = 0.71 (ISSR)] (Shahnawaz *et al*., 2011).

Based on the dendogram generated by UPGMA, most of the populations branched according to their geographical locations except the population of Uttara Kannada, which branched with Kolhapur and Tilari population (ISSR). PCoA also displayed similar patterns where Uttara Kannada clustered separately in two segments with first 16 accessions in one cluster and next 17 in other cluster (ISSR). The formation of 5 different clusters indicates the existence of 5 geographically distinct populations. These 5 different populations might have arisen through 5 different clones.

Our study showed a relatively high level of genetic diversity at species level, ISSR (h = 0.27, I = 0.42) and RAPD (h = 0.26, I = 0.41). WG population showed a relatively high level of genetic diversity {ISSR (h = 0.29, I = 0.44) and RAPD (h = 0.31, I = 0.48)} compare to that of EI population {ISSR (h = 0.17, I = 0.26) and RAPD (h = 0.29, I = 0.44)}.

The low within-population genetic diversity in EI could be due to its geographic isolation and high distance between the populations. A study carried out with 22 accessions of *G. sylvestre* (Shahnawaz *et al*., 2011) generated lower values (h = 0.18, I = 0.26) than our results. In our study, the values of *h* and *I* within population ranged from 0.04 to 0.18, and 0.06 to 0.27 respectively. The higher values at species level for these parameters might be due to the larger geographical distance between EI and WG.

Conclusion

The present study is first of its kind where we have studied the genetic diversity analysing a larger number of samples and covered a wide range of geographical area using various molecular tools (POPGENE, GenAlEx and TFPGA) compare to other studies done in *G.sylvestre*.

The study showed that there is high genetic differentiation and the low gene flow in this species of plant, which suggests that conservation should emphasis on preserving the populations. The present study provides significant information on the pattern of genetic variation and diversity which will help the concerned authorities in designing strategies on the conservation of *G. sylvestre*.

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