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Assessment of Genetic Diversity among *Rauvolfia serpentina* Accessions Using Random Amplified Polymorphic DNA (RAPD) Markers

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ABSTRACT

Rauvolfia serpentina is an important medicinal herb since it contains medicinally important compounds. In the present study, the genetic diversity among *R. serpentina* accessions collected at different geographical regions in India was assessed through random amplified polymorphic DNA (RAPD). Totally 19 accessions of *R. serpentina* were screened with 60 RAPD primers of which 15 (25%) primers were found to be the most informative and produced multiple band profiles with a number of amplified DNA fragments varying from 7 to 11. Of the 143 amplified bands, 95 were polymorphic (66.46%), with an average of 6.33 polymorphic fragments per primer. Jaccard's genetic similarity co-efficient varied from 0.313 to 0.875. A UPGMA dendrogram showed two main clusters split at Jaccard's similarity co-efficient of 0.313. The information obtained here could be valuable for devising strategies for conservation of this medicinal plant.

Keywords: dendrogram, genetic diversity, UPGMA

INTRODUCTION

Rauvolfia serpentina is a small, woody shrub which originated in South Asia commonly known as Indian Snakeroot or Sarpagandha belongs to the family of Apocynaceae. It is a medicinally famous herb in Ayurveda, Siddha, Unani and Western system of medicines (Qureshi *et al*. 2009). *R. serpentina* holds an important position in the pharmaceutical world because it contains number of pharmaceutically important compounds including reserpine and serpentine. The oleoresin fraction of roots is rich in reserpine and widely used as anti-hypertensive agent. The plant is extensively used in the treatment of insanity and snake bite. The alkaloid reserpine from root extract is used to treat high blood pressure and mental disorders including schizophrenia (Isharwal and Gupta 2006). The root extract of this plant is very useful in disorders of gastro intestinal tract viz., diarrhea, dysentery, cholera and colic (Qureshi *et al*. 2009). The root is also used to stimulate uterine contractions and is recommended for use in difficult childbirth cases (Villar *et al*. 1998).

Random amplified polymorphic DNA (RAPD) markers were successfully employed to discriminate the genetic polymorphism in several Apocynaceae family medicinal plants including *Catharanthus roseus* (Shaw *et al*. 2009), *Dyera costulata* (Poerba *et al*. 2009), *Mandevilla velutina* (Bertoni *et al*. 2010), *Thevetia* and *Alstonia* (Mahmood *et al*. 2011). In addition, RAPD markers were successfully employed to estimate the genetic diversity in important medicinal plants such as *Lippa sidoides*. (Viccini *et al*. 2004), *Zingiber officinales* (Palai and Rout 2007), *Costus speciosus* (Mandal *et al*. 2007), *Tribulus terrestris* (Sarwat *et al*. 2008), and *Jatropha* spp. (Ram *et al*. 2008). Padmalatha and Prasad (2007) successfully applied RAPD markers to study the genetic diversity in *R. serpentina* accessions collected from six locations of Andhra Pradesh, India. Goel *et al*. (2009) also used RAPD markers to analyze the genetic fidelity of micropropagated *R. serpentina* plants. However, there is much limited information available on the RAPD markers in *R. serpentina* although the available reports are only concerned with very few accessions collected from a limited area. Hence, the present investigation was carried out with the objective of studying the genetic diversity in 19 accessions of *R. serpentina* collected from different locations throughout India.

MATERIALS AND METHODS

Plant material

Nineteen accessions of *R. serpentina* were used in the present investigation. Leaf samples (fifth leaf from top) from one-year-old flowering plants of each accession were collected from natural populations growing in various populations in India (**Table 1**). Immediately after harvesting the leaf samples, thoroughly washed with distilled water, dried with silica gel and stored at -20°C prior to genomic DNA isolation.

Genomic DNA isolation

All the chemicals used for the genomic DNA isolation such as Tris, HCl, EDTA, NaCl, CTAB, β-mercaptoethanol, PVP, phenol, chloroform, isoamyl alcohol, isoproponol, ethanol and RNase A were procured from Sigma (St. Louis, MI, USA). Genomic DNA was extracted from harvested leaf samples of *R. serpentina* by adopting the procedure outlined by Dellaporta *et al*. (1983) with minor modifications. The *R. serpentina* leaf tissue was powdered in liquid nitrogen and immediately transferred to a micro-centrifuge tube containing 1 ml of extraction buffer (100 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 1.5 M NaCl, 2% CTAB, 0.2% β-mercaptoethanol (v/v) and 1% PVP (w/v) and mixed well to form a slurry and incubated at 65°C for 60 min. An equal volume of chloroform: isoamylalcohol (24: 1 v/v) was added to the extract prior to centrifugation at 12,000 rpm for 12 min at room tempera-

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Table 1 List of 19 accessions of *Rauvolfia serpentina* used in the genetic diversity analysis using RAPD markers.

Accession No	Place of collection in India	Geographical location
RW01	Coorg, Karnataka	12° 25' N, 75° 45' E
RW02	Barmer, Rajasthan	25° 45' N, 71° 25' E
RW03	Loktak, Manipur	24° 30' N, 93° 55' E
RW04	Ballia, Uttar Pradesh	25° 44' N, 84° 11' E
RW05	Balaghat, Madya Pradesh	21° 48' N, 80° 15' E
RW06	Sibsagar, Asaam	26° 59' N, 94° 38' E
RW07	Dhubri, Asaam	26° 02' N, 89° 58' E
RW08	Alleppey, Kerala	09° 30' N, 76° 23' E
RW09	Kangra, Himachal Pradesh	32° 05' E, 76° 18' E
RW10	Dhanbad, Jharkhand	23º 47' N, 86º 30' E
RW11	Jaintia Hills, Meghalaya	25° 30' N, 91° 30' E
RW12	Udaipur, Tripura	23° 31' N, 91° 31' E
RW13	Naga Hills, Nagaland	26° 00' N, 94° 20' E
RW14	Marmagao, Goa	15° 25' N, 73° 43' E
RW15	Kolli hills, Tamilnadu	10° 12' N, 77° 56' E
RW16	Darjilling, West Bengal	27° 03' N, 88° 18' E
RW17	Kansal, Chandigarh	30° 44' N, 76° 47' E
RW18	Hoshiarpur, Punjab	31° 32' N, 75° 57' E
RW19	Gaya, Bihar	42º 49' N, 85º 01' E

ture. To the supernatant equal volume of isopropanol and half volume of 5 M NaCl were added and incubated at room temperature for 30 min followed by centrifugation (Remi, Mumbai, India) at 12,000 rpm for 12 min at room temperature. The pellet was washed with 70% ethanol by centrifuging at 12,000 rpm for 12 min. Dried DNA pellet was dissolved in Tris-EDTA (TE) buffer and 3 µl of RNase A (10 mg/ml) was added and incubated for 1 h at 37°C, followed by extraction twice with chloroform: Isoamyl-alcohol (24: 1) by centrifugation at 12,000 rpm for 10 min at room temperature. To the aqueous phase cold absolute ethanol was added and DNA was collected in the form of pellet by centrifuging at 12, 000 rpm for 10 min followed by 70% ethanol washing twice, air dried and the DNA was dissolved in 100 µl of TE buffer. Five aliquots of genomic DNA isolated from five plants of each genotype were pooled together and quantified by measuring absorbance at 260 nm using Chemito UV – 2600 spectrophotometer (Chemito Instruments, Mumbai, India) and stock DNA was diluted to make a working solution of 10 ng/µl for RAPD–PCR analysis.

RAPD-PCR

A total of 60 arbitrary primers from Operon RAPD kits A, B, C, D, E, and F (Operon Technologies Inc., Alameda, CA, USA) were used to generate preliminary RAPD profiles. Amplifications were performed in a 25-µl reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl (SRL Pvt. Ltd, Mumbai, India), 2.5 mM $MgCl_2$ (SRL Pvt. Ltd, Mumbai, India), 0.01% gelatin (SRL Pvt. Ltd, Mumbai, India), 0.2 mM each dNTP (Genei, Bangalore, India), 20 pmol RAPD primer, 50 ng genomic DNA, and 0.5 U *Taq* DNA polymerase (Genei, Bangalore, India). Amplifications were performed in an Eppendorf Master Cycler gradient (Eppendorf Netheler-Hinz, Hamburg, Germany). Amplification conditions were an initial denaturation at 94°C for 4 min and 45 cycles at 94°C for 1 min, 37°C for 1 min, 72°C for 2 min, followed by 5 min at 72°C. Amplified products were separated on 1.5% agarose gel in 1× TBE buffer (100 mM Tris-HCl, pH 8.3, 83 mM boric acid, 1 mM EDTA) at 50 V. The gels were stained with 0.5 µg/ml ethidium bromide solution and visualized by illumination under UV light and photographed under ultraviolet light using a Gel Doc 2000 (Bio-RAD). Molecular weights of amplified bands were estimated using 100 bp plus DNA ladder (Fermentas Life Sciences, Maryland, USA).

Data analysis

The PCR reactions were repeated three times, using the same conditions to check the reproducibility of the selected primers for 19 DNA samples (genomic DNA from five plants of same genotype were pooled together). RAPD markers across 19 accessions were visually scored for the presence (1) or absence (0) for each primer. Only distinct, well-resolved and unambiguous bands were scored, discarding faint bands. The results were analyzed based on the principle that a band is considered to be 'polymorphic' if it is present in some individuals and absent in others, and 'monomorphic' if present in all the individuals. All amplifications were repeated thrice and only reproducible bands were considered for scoring. The amplified fragments were scored as (1) at the presence and (0) at the absence of homologous bands. Data analyses were conducted using the NTSYS-pc2.10e software according to its manual (Rohlf 2000). Dendrograms were constructed with the method of unweighted pair group method with arithmetic mean (UPGMA) based on the similarity matrices calculated with Jaccard coefficient.

RESULTS AND DISCUSSION

Identification and quantification of the variation within and between populations is essential for the establishment of effective and efficient conservation for plants. Morphological and molecular markers have been proved to be valuable markers to analyze the genetic diversity in many medicinally important plants. Morphological characters in plants may be affected by environmental conditions and a plant grown in different climatic conditions may be morphologically different. Thus, the use of morphological characters for classification may result in discrepancy (Singh *et al*. 2011). Hence, molecular markers came into light to overcome the flaws of using morphological characters to analyze the similarity/diversity within and among the species. The efficiency of a molecular marker technique depends on the amount of polymorphism it can detect among set of accessions under investigation. The utility of RAPD markers in estimating genetic diversity has been demonstrated and proved to be possible within *Rauvolfia* species (Padamalata and Prasad 2006, 2007; Mahesh *et al*. 2008). However, limited work has been carried out and only with few *R. serpentina* cultivars. Hence, in the present investigation, we established efficient RAPD markers to analyze the genetic polymorphism among 19 accessions of *R. serpentina* collected from different locations in India (**Table 1**).

Genomic DNA (10 µg) was isolated from 3.0 g of fresh tender leaf tissues of *R. serpentina* by following the modified CTAB method (Dellaporta *et al*. 1983). RAPD analysis of *R. serpentina* genomic DNA was carried out under optimized amplification conditions which were identical for all the reactions. All amplification reactions were performed in triplicate and only highly reproducible bands were considered for analysis. Of the 60 RAPD primers initially were screened, only 15 primers (25%) produced sharp reproducible intensely stained fragments that could be scored for the genetic diversity analysis in *R. serpentina*.

Table 2 Salient features of the amplification products obtained in 19 *Rauvolfia serpentina* genotypes with 15 primers using RAPD analysis.

Primer name	Primer sequence	Total no. of bands	No. of polymorphic bands	Percentage of polymorphic bands*
OPA 02	5' TGCCGAGCTG 3'	09	05	55.55
OPA 06	5' AGGGGTCTTG 3'	11	07	63.63
OPA 08	5' GTGATCGCAG 3'	10	06	60.00
OPA 19	5' TCTGTGCTGG 3'	10	09	90.00
OPA 20	5' AGGTGACGCT 3'	09	06	66.66
OPB 10	5' CTGCTGGGAC 3'	08	05	62.50
OPB 16	5' TTTGCCCGGA 3'	11	07	63.63
OPB 18	5' CCACAGCAGT 3'	10	05	50.00
OPD 08	5' GTGTGCCCCA 3'	07	05	71.42
OPD 16	5' AGGGCGTAAG 3'	09	06	66.66
OPC 03	5' GGGGGTCTTT 3'	08	05	62.50
OPC 08	5' TGGACCGGTG 3'	10	08	80.00
OPC 15	5' GACGGATCAG 3'	10	07	70.00
OPE 01	5' CCAAGGTCCC 3'	11	07	63.33
OPF 12	5' ACGGTACCAG 3'	10	07	70.00

*Percentage of polymorphic bands = No. of polymorphic bands / total number of bands X 100 (Subramanyam *et al*. 2012)

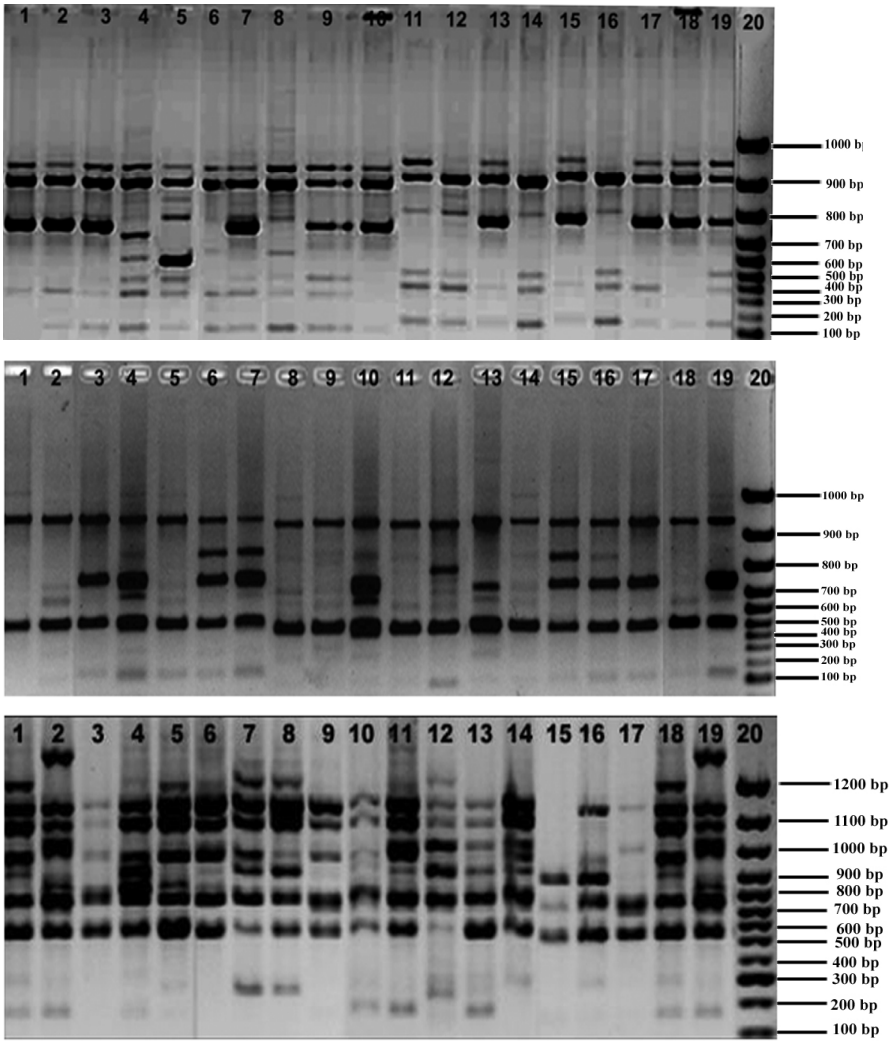

Fig. 1 RAPD polymorphism in *Rauvolfia serpentina* genotypes detected with OPA-19, OPD 08, and OPC 08 RAPD primers.

Table 3 Jaccard's similarity coefficient of 19 *Rauvolfia serpentina* (RW) accessions.

	RW1	RW2	RW3	RW4	RW5	RW6	RW7	RW8	RW9	RW10	RW11	RW12	RW13	RW14	RW15	RW16	RW17	RW18	RW19
RW1	1																		
RW2	0.688	1																	
RW3	0.571	0.5	1																
RW4	0.474	0.5	0.647	1															
RW5	0.529	0.55	0.529	0.684	1														
RW6	0.563	0.579	0.786	0.722	0.611	1													
RW7	0.5	0.6	0.688	0.65	0.632	0.875	1												
RW8	0.529	0.632	0.529	0.6	0.765	0.611	0.722	1											
RW9	0.563	0.579	0.563	0.722	0.706	0.647	0.579	0.706	1										
RW10	0.667	0.5	0.563	0.632	0.526	0.556	0.5	0.526	0.647	1									
RW11	0.643	0.556	0.533	0.45	0.5	0.529	0.474	0.588	0.625	0.625	1								
RW12	0.444	0.55	0.368	0.391	0.5	0.381	0.476	0.579	0.526	0.381	0.5	1							
RW13	0.647	0.65	0.647	0.7	0.524	0.632	0.571	0.455	0.632	0.632	0.526	0.524	1						
RW14	0.571	0.588	0.467	0.474	0.444	0.471	0.421	0.444	0.563	0.389	0.533	0.733	0.647	1					
RW15	0.5	0.368	0.615	0.5	0.389	0.6	0.529	0.389	0.5	0.6	0.375	0.25	0.5	0.313	1				
RW16	0.438	0.4	0.769	0.611	0.588	0.625	0.556	0.5	0.625	0.529	0.6	0.5	0.611	0.533	0.467	1			
RW17	0.615	0.444	0.75	0.588	0.471	0.6	0.529	0.471	0.6	0.714	0.467	0.316	0.588	0.4	0.818	0.571	1		
RW18	0.846	0.706	0.5	0.5	0.556	0.5	0.526	0.556	0.588	0.688	0.563	0.474	0.667	0.5	0.533	0.389	0.643	1	
RW19	0.588	0.6	0.5	0.571	0.476	0.5	0.455	0.348	0.5	0.579	0.474	0.476	0.833	0.588	0.444	0.556	0.529	0.611	1

Table 2 represents the number of amplified fragments and the degree of polymorphism revealed by each RAPD primer. All the chosen 15 primers showed amplification with the DNA of 19 accessions. The number of amplified fragments varied from seven (OPD 08) to eleven (OPA 06, OPB 16, and OPE 01), which varied in size from 125 to 2,322 bp. Of the 143 amplified fragments, 95 were polymorphic (66.43%), with an average of 6.33 polymorphic fragments/primer. Percentage polymorphism ranged from 50% (OPB 18) to a maximum of 90% (OPA 19). Only four out of 15 primers showed more than 70% polymorphism. **Fig. 1** is the representative of the extent of polymorphism observed among the *R. serpentina* accessions as revealed by OPA 19, OPD 08, and OPC 08. Similar to the obtained results, Padmalatha and Prasad (2007) reported 70% polymorphism among *R. serpentina* accessions collected from

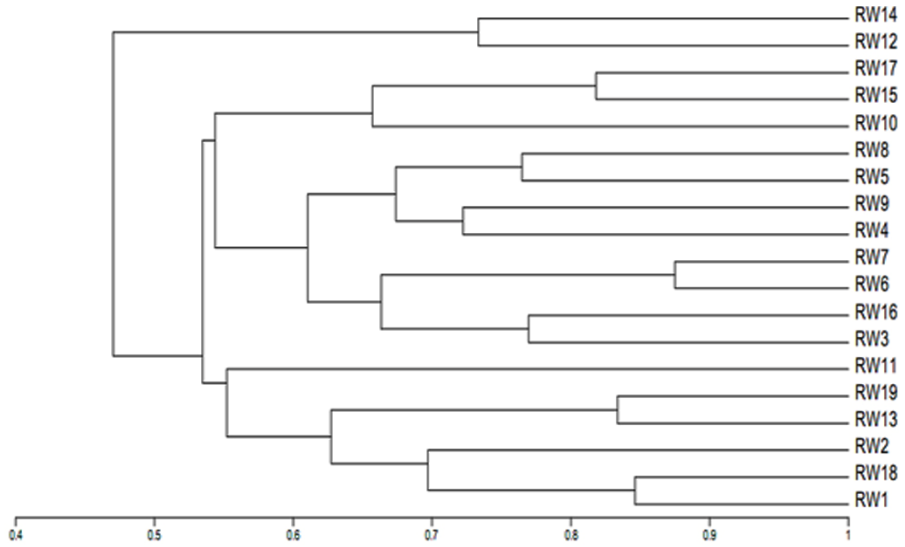

Fig. 2 Dendrogram constructed for *Rauvolfia serpentina* from different locations in India based on Jaccard's similarity coefficient. RW indicates *R. serpentina.*

Table 4 Genetic polymorphism analysis in different medicinal plants using RAPD markers.

Medicinal plant name	No. of samples tested	No. of RAPD primers used	No. of RAPD primers responded	Total no. of bands	Total no. of polymorphic bands	% poly-morphism	Research group
Lippa spp.	11	30	18	490	489	99.79	Viccini *et al*. 2004
Rauvolfia tetraphylla	05	08	05	397	205	51.63	Padmalatha and Prasad 2006
Rauvolfia serpentina	06	40	23	379	263	70	Padmalatha *et al*. 2007
Zingiber officinale	08	40	12	55	25	45.45	Palai and Rout 2007
Costus speciosus	14	12	04	105	77	73.33	Mandal *et al*. 2007
Catharanthus roseus	14	25	14	56	46	82	Shaw *et al*. 2008
Tribulus terrestris	24	21	21	276	163	59.05	Sarwat *et al*. 2008
Jatropha spp.	12	26	18	134	112	80.2	Ram *et al*. 2008
Trichodesma indicum	50	80	20	125	121	96.8	Verma *et al*. 2009
Dyera costulata	08	71	34	864	784	90.74	Poerba and Widjaya 2009
Mandevilla velutina	67	100	11	111	92	82.88	Bertoni *et al*. 2010
Bacopa monnieri	25	25	10	110	14	12.72	Karthikeyan *et al*. 2011

six different locations. Alam *et al*. (2009) reported 50-100% of polymorphism in *Podophyllum hexandrum* by using RAPD markers. In contrast, Rawashdeh (2011) reported 13-16.4% polymorphism in *Achillea fragrantissima* and Verma *et al*. (2009) analyzed the genetic diversity in *Trichodesma indicum* by using RAPD markers and produced 0.14-1.0% of polymorphism.

Jaccard's genetic similarity co-efficient varied from 0.313 to 0.875 (**Table 3**). The highest genetic similarity of 0.875 was recorded between RW 6 and RW 7, while the lowest of 0.313 was between RW 14 and RW 15. UPGMA cluster analysis of the Jaccard's similarity coefficient generated a dendrogram (**Fig. 2**) which illustrated the overall genetic relationship among the accessions studied. UPGMA dendrogram showed two main clusters with a split at Jaccard's similarity co-efficient of 0.313. Cluster I include two accessions - RW12 and RW14. Cluster II was divided into two sub clusters. Sub cluster A contain 6 accessions namely RW 1, RW 18, RW 2, RW 13, RW 19, RW 11, whereas sub cluster B again subdivided into cluster C which contains 8 accessions namely RW 3, RW 16, RW 6, RW 7, RW 4, RW 9, RW 5, RW 8 and cluster D contains remaining three accessions (RW 10, RW 15, RW 17) (**Fig. 1**).

The dendrogram developed based on the Jaccard's similarity coefficient (UPGMA) revealed that the collected *R. serpentina* accessions were highly differentiated by their own genetic distance. The clustering results of different accessions suggest that *R. serpentina* undergoes major part of genetic variation by environmental factors. The wide range of variation observed might also be due to two evolutionary forces, which include pollen flow and local selection pressures. Pollen can be dispersed over large distances; this long-term reciprocal movement of pollen must also have contributed to the variation. Recent experiments using pollen traps have shown that oak pollen can migrate at seven kilometers (Lahtinen *et al*. 1996). The local selection pressures may be due to the effects of environmental factors and due to struggle for existence in nature. The wide spread occurrence of the wind pollination and breeding systems that promotes out crossing may lead to higher genetic diversity.

RAPD markers have been used successfully employed to study the genetic diversity in *R. serpentina* and several Apocynaceae family medicinal plants including *Catharanthus roseus* and *Dyera costulata* where 82 and 90.74% polymorphism was observed among 14 (*Catharanthus roseus*) and 8 (*Dyera costulata*) accessions, respectively (Shaw *et al*. 2009; Poerba and Widjaya 2009). In addition RAPD markers also used to study the genetic diversity in *Zingiber officinale* (Palai and Rout 2007), *Costus speciosus* (Mandal *et al*. 2007), *Tribulus terrestris* (Sarwat *et al*. 2008), *Jatropha* spp., (Ram *et al*. 2008), *Trichodesma indicum* (Verma *et al*. 2009), *Mandevilla velutina* (Bertoni *et al*. 2010), and *Bacopa monnieri* (Karthikeyan *et al*. 2011) (details in **Table 4**). Although the results of the present study provide evidence for genetic loss, information is required on the implications of reduced genetic variation for survival and fertility. The high degree of genetic variation or differentiation recorded by the transfer of germplasm between different locations should be avoided, to ensure that the genetic material is adapted to local conditions (Ennos 1994). The genetic analyses presented here could be

used for the development of conservation strategies for the species, for example through the description of appropriate units of management (Newton *et al*. 1999). In the case where gene flow between neighboring populations is not limited, populations of longer geographical distances will generally show greater diversification. Hence the grouping of these accessions is independent of the geographical distance (Mahesh *et al.* 2008). It proved that the accessions collected from different locations exhibited similarities (leaf morphology and length, color of the petiole and midrib, flower morphology and fruit morphology) but their RAPD fingerprinting differed markedly. The more variation, the better the chance that at least some of the individuals will have an allelic variant that is suited for the new environment, and will produce offspring with the variant that will in turn reproduce and continue the population into subsequent generations (Mahesh *et al.* 2008). In order to prevent depletion of biodiversity due to man-made efforts or otherwise, it is necessary to understand how the diversity of life particularly in the genetic level is maintained under natural conditions. Based on this knowledge one can suggest appropriate strategies and policies for the conservation of biodiversity. An understanding of the genetic diversity responsible for individual species adaptations and responses to their environment (intraspecific diversity) is a foundation for understanding almost all ecological and evolutionary processes (Mahesh *et al.* 2008). Further analysis is necessary to find out the individual polymorphism in each population and to be the best and this data may be correlated with other population and the superior population can be identified. Therefore the difference found in the dendrogram could be partially explained by different number of loci and there coverage of overall genome in obtaining reliable estimation of genetic relationships among the plant species of *R. serpentina*.

CONCLUSION

Analysis of RAPD could be useful to detect genetic differentiation of *R. serpentina* among 19 locations in India. From the work done in this medicinal plant collected at different geographical locations, it was understood that each location varied with respect to environmental factors and genetic parameters. Thus by analyzing the genetic variability we are able to identify the elite population. The information obtained here could be valuable for devising strategies for conservation of *R. serpentina*.

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