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Genetic Diversity in *Rauvolfia tetraphylla* L.f using RAPD Markers

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Abstract

The present study is the first report of molecular variations in different accessions of *Rauvolfia tetraphylla* L.f, a medicinally important plant collected from seven locations of Andhra Pradesh, India. Molecular analysis was carried out using RAPD markers. Out of the 40 primers screened from OPA and OPC Kits, a total of 205 scorable polymorphic markers out of 397 total markers were generated. Polymorphism of 51.6% was found with 3 unique markers. Levels of genetic diversity within accessions i.e., the genetic distance ranged from 0.816-0.932. Cluster analysis based on Dice coefficient showed two major groups indicating that mostly in cross-pollinated plants, high levels of differentiation among accessions exists independent of geographical distance. Hence the results of the present study can be seen as a starting point for future researches on the population and evolutionary genetics of this species. Understanding such variation would also facilitate their use in various conservational management practices, rootstock breeding and hybridisation programmes.

Key words: Conservation, Genetic diversity, Polymerase chain reaction, Randomly Amplified Polymorphic DNA, *Rauvolfia tetraphylla*

Introduction

Rauvolfia tetraphylla L. (Ap ocynaceae) is a, small evergreen woody shrub. It is native to West Indies and introduced to India where it is cultivated in gardens in Uttar Pradesh, West Bengal, Tamil Nadu and Kerala. It has become naturalized in many localities and is distributed in moist habitats in Andhra Pradesh (A.P). Pharmacologically the roots are important although they reportedly contain low concentrations of the alkaloids like reserpine, ajmalicine, and tetraphyllicinine, posseses hypotensive property and can cure many other diseases (Faisal et al., 2005). The root of this plant is used as substitute of *R. serpentina* for alkaloid contents. Medicinally the plant extract mixed with castor oil is used in treatment of refractory ailments and bark decoction is used for chronic refractory skin diseases and destroys (parasites (Parrotta, 2001). The root is also used to stimulate uterine contractions and is recommended for use in difficult childbirth cases.

The platelet antiaggregant activity of 17 aqueous extracts of plants traditionally used in Guatemala for the treatment of 'blood disorders' and parasitic infections were assayed where *R. tetraphylla* was one of them (Villar et al., 1998) The government of India has restricted the export of its drugs to reduce its exploitation and thus conserve natural stand. This has resulted in a shortage of its alkaloids in the world market. For conservation of this plant species studies on micropropagation were carried out by using nodal segments (Faisal and Anis, 2002; Faisal et al., 2005). So far studies on genetic diversity analysis has not been carried out at any level i.e., at morphological, biochemical or at the molecular level which is most important from the conservation point of view.

RAPD markers have been employed as an alternative for morphological and biochemical markers (Dawson et al., 1993; Yoon and Glawe, 1993; Pei et al., 1995; Wolfe and Liston,; 1998;, Su et al., 1999; Esselman et al., 2000). The genetic diversity can be explained by the aid of calculation of polymorphism levels and cluster diagram. RAPD markers provide equivalent levels of resolutions for determining genetic relationships (Santo et al., 1994). Reliability of RAPDs among closely related taxa and the limitation of RAPD data for producing expected associations among more divergent taxa were observed in *Pisum* species (Hoeyet al., 1996). Thus the present study was concentrated to determine the genetic diversity between different accessions of *R. tetraphylla* collected from Andhra Pradesh.

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Table 1. *Rauvolfia tetraphylla* germplasm accessions collected from different locations of Andhra Pradesh used for molecular diversity analysis.

Acc.No	Location	District	Latitude	Longitude
1.RTMR	Mallur	Warangal	18 °15'	80 °31'
2.RTTI	Tirupathi	Chittoor	13 °10'	77 °20'
3.RTBY	Balpally	Kadapa	14 °25'	79 °18'
4.RTHC	Hyderabad	Hyderabad	27 °20'	78 °20'
5.RTDY	Dulapally	Ranga reddy	27 °20'	78 °20'
6.RTVA	Vijayawada	Krishna	15 °09'	75 °14'
7.RTMI	Maredumilli	Khammam	19 °35'	80 °48'

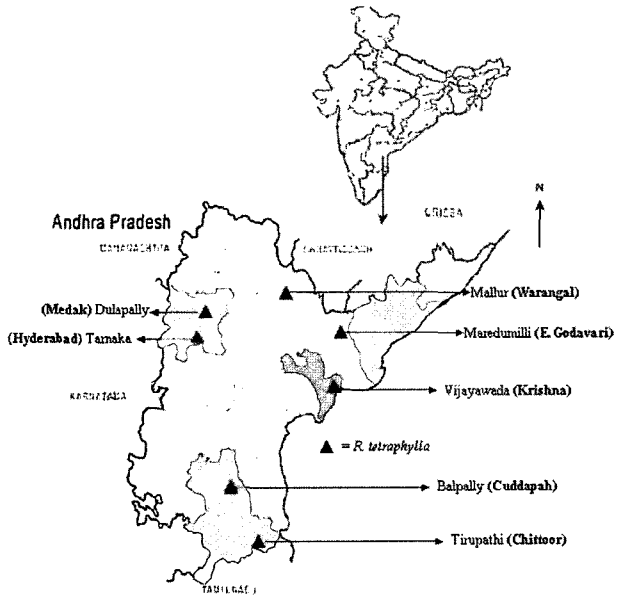

Figure 1. Locations in Andhra Pradesh from where the germplasm of *Rauvolfia tetraphylla* L.f was collected from various locations (Districts are mentioned in the parenthesis).

Materials and methods

Plant Source

The plant material of *Rauvolfia tetraphylla* was collected from various locations from A.P, India. The plants were identified based on the flora of A.P (Pullaiah and Chennaiah, 1998). A total of seven accessions were collected from Mallur (Warangal), Tirupathi (Chittoor), Balpally (Cuddapah), Hyderabad (Hyderabad), Dulapally (Rangareddy), Vijayawada (Krishna) and Maredumilli (East Godavari) for molecular diversity analysis. District names are given in the parenthesis. The collection details are depicted topographically in Figure 1. The latitude and longitude of locations of collection is mentioned in Table 1.

Isolation of genomic DNA

DNA was isolated by using CTAB protocol developed by Doyle and Doyle (1987) with slight modifications. Young leaf tissue (3 gm) was ground into a fine powder in liquid Nitrogen along with 0.1 gm of PVPP and transferred to preheated 2% CTAB extraction buffer (2% CTAB, 100 mM Tris HCl, 20 mM EDTA, pH 8.0, 1.4 M NaCl) containing 10 mM β-mercaptoethanol per gram of tissue. The slurry was incubated for 90 min at 65°C in water bath. An equal volume of chloroform: isoamylalcohol (24:1) was added to the extract prior to centrifugation at 12,000 rpm for 15 min at 4°C. To the supernatant equal volumes of ice cold isopropanol was added and incubated at -20°C for a period of minimum of 30 min or overnight followed by centrifugation at 12,000 rpm for 15 min at 4°C. The pellet was collected and washed with 70% ethanol, centrifuged at 10,000 rpm for 8 min. The pellet was dried and redissolved in 100µl of TE buffer.

In order to eliminate RNA contamination, the sample was treated with 5.0 µl of RNase A (10 µg/µl), incubated at 37°C overnight. This was followed by phenol: chloroform: isoamylalcohol (24:1:1) extraction and centrifugation at 8000 rpm for 15 min. To the supernatant equal volumes of chloroform: isoamylalcohol (24:1) was added and centrifuged at 12,000 rpm for 15 mins. To the supernatant 1/10th volume of 3 M sodium acetate and equal volumes of ice-cold isopropanol was added and left for a mimimum duration of 30 min or overnight at -20°C to precipitate DNA followed by centrifugation at 12,000 rpm for 15 min. The DNA was washed by adding 70% ethanol and centrifuged at 10,000 rpm for 10 min at 4°C (Sambrook et . al., 1989). After complete drying the pellet was dissolved in TE buffer depending on the pellet size and was stored at -20°C for future use.

Qualitative and quantitative extraction of DNA

To test the quality of DNA, the OD values were recorded at 260 and 280 nm and the ratio of OD 260 to OD 280 was calculated to check the purity of each DNA sample which was between. 1.7 and 1.8. Further purity of DNA,

was tested gel by agarose gel electrophoresis, using 0.8% TBE- agarose. Gels were stained with ethidium bromide and viewed on a UV transilluminator, photographed with the help of a gel documentation system (LTF Labortechnik, Germany). DNA was also quantified based on the spectrophotometric measurements of UV absorption at 260 nm, assuming 1.0 OD at 260 nm is equal to 50 µg of DNA (Sambrook et al., 1989) and the concentrated DNA was diluted with ultrapure Milli Q (Milli Q academic) sterile water to 50 ng/µl.

RAPD analysis

Forty decamer primers of arbitrary sequence (Kits A and C provided by Operon Technologies Inc, Alameda, CA) were tested for PCR amplification among seven accessions of *R. tetraphylla*. The analysis involved 2 steps a) PCR amplification and annealing of single arbitrary primer at random on the total genome and b) a Agarose gel electrophoresis. PCR reactions were carried out in a DNA Thermocycler (MJ Research Inc. USA.) with a heated lid. The PCR ingredients and conditions were followed accordingly (Padmalatha and Prasad, 2006). Negative controls were also run without template DNA to ensure the amplification. PCR products were electrophoresed on 2% (W/V) agarose gels, in 1X TBE Buffer at 50 V for 3 hrs and then stained with ethidium bromide (0.5 µg/ml). Gels with amplification fragments were visualized and photographed under UV light. Lambda DNA *EcoR* 1 *Hind* 111 double digest was used as molecular marker (Genetics, New Delhi, India) to determine the size of the fragments.

Data scoring and analysis

For each accession, polymorphism was scored as 1 for the presence and 0 for absence of a band. RAPD data generated with forty primers was used to compile a binary matrix for cluster analysis using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, Biostatistics, New York, USA, software version 2.02j package), (Rohlf, 1998). Genetic similarity among accessions was calculated according to Dice similarity coefficient (Dice, 1945). The similarity coefficients were then used to construct a dendrogram using the UPGMA (Unweighted Pairwise Group Method with Arithmetical averages) through NTSYS-pc. The screening of the entire set of samples was done thrice to assess repeatability of the RAPD profiles and identical RAPD patterns were obtained.

Results

Analysis of seven accessions of *Rauvolfia. tetraphylla* with a total of forty primers (OPA and OPC series) revealed 51.6% of polymorphism. The number of scorable polymorphic markers generated was 205 out of 397 total markers (Table 2). The numbers of unique markers generated were very few (4). The primer with maximum number of polymorphic bands is OPC-3 (14 bands) (Figure 2a), the primer with minimum number of polymorphic bands is OPA-17 (1 band) (Figure 2b) and the primer, which did not show any polymorphism is OPA-12 (Figure 2c) i.e., which exhibited complete monomorphism. Hence the range of the bands generated for all the primers falls between 0-14. There is no primer, which did not exhibit amplification. The GC% of all the primers ranged from 60-70%, did not

Table 2. Polymorphism among seven accessions of *R. tetraphylla*

Primer Code	Primer sequence 5'-3'	TB	PB	% polymorphism
OPA-01	CAGGCCCTTC	13	5	38.4
OPA-02	TGCCGAGCTG	4	4	100
OPA-03	AGTCAGCCAC	12	5	41.6
OPA-04	AATCGGGCTG	13	8	61.5
OPA-05	AGGGGTCTTG	9	4	44.4
OPA-06	GGTCCCTGAC	12	5	41.6
OPA-07	GAAACGGGTG	13	2	15.3
OPA-08	GTGACGTAGG	14	3	21.4
OPA-09	GGGTAACGCC	14	11	78.5
OPA-10	GTGATCGCAG	10	6	60.0
OPA-11	CAATCGCCGT	15	11	73.3
OPA-12	TCGGCGATAG	3	0	0
OPA-13	CAGCACCCAC	14	8	57.1
OPA-14	TCTGTGCTGG	7	2	28.5
OPA-15	TTCCGAACCC	5	2	40.0
OPA-16	AGCCAGCGAA	7	4	57.1
OPA-17	GACCGCTTGT	6	1	16.6
OPA-18	AGGTGACCGT	11	2	18.1
OPA-19	CAAACGTCGG	5	2	40.0
OPA-20	GTTGCGATCC	11	2	18.1
OPC-01	TTCGAGCCAG	5	5	100
OPC-02	GTGAGGCGTC	9	4	44.4
OPC-03	GGGGGTCTTT	14	14	100
OPC-04	CCGCATCTAC	14	6	42.8
OPC-05	GATGACCGCC	15	5	33.3
OPC-06	GAACGGACTC	12	5	41.6
OPC-07	GTCCCGACGA	10	7	70.0
OPC-08	TGGACCGGTG	12	7	58.3
OPC-09	CTCACCGTCC	6	3	50.0
OPC-10	TGTCTGGGTG	12	4	33.3
OPC-11	AAAGCTGCGG	15	13	86.6
OPC-12	TGTCATCCCC	10	3	30.0
OPC-13	AAGCCTCGTC	12	11	91.6
OPC-14	TGCGTGCTTG	7	3	42.8
OPC-15	GACGGATCAG	10	3	30.0
OPC-16	CACACTCCAG	10	5	50.0
OPC-17	TTCCCCCCAG	4	4	100
OPC-18	TGAGTGGGTG	17	11	64.0
OPC-19	GTTGCCAGCC	4	2	50.0
OPC-20	ACTTCGCCAC	11	3	27.2

Note: TB-Total bands, PB-Polymorphic bands

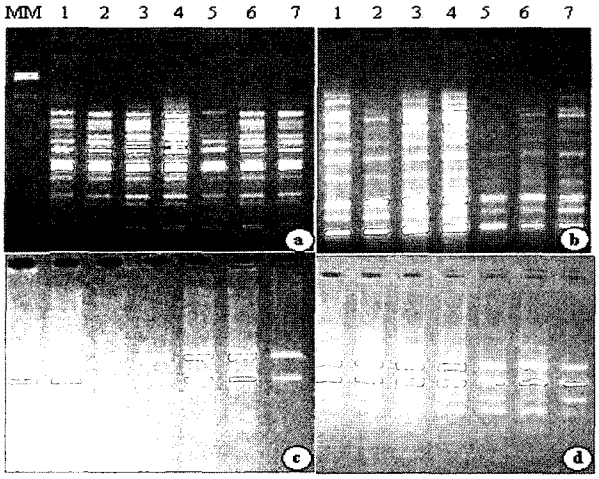

Figure 2. a) RAPD profile using primer OPC-03 (5' GGGGGTCTTT 3') Mostly *monomorphic markers are generated among* the accessions of *R. tetraphylla* but unique species specific polymorphic bands are generated in *R. tetraphylla*. b) RAPD profile using primer OPA-17 (5 GACCGCTTGT 3') Polymorphic markers are generated in case of accessions in *R. tetraphylla*. c) RAPD profile using primer OPA-12 (5' TCGGCGATAG 3'). Monomorphism is observed among the accessions of *R. tetraphylla*. d) RAPD profile using primer OPC-02 (5' GTGAGGCGTC 3'). In case of accessions in *R. tetraphylla* polymorphic markers are generated.

	1	2	3	4	5	6	7
RTMR	1.000						
RTTI	0.915	1.000					
RTBY	0.888	0.893	1.000				
RTHC	0.880	0.885	0.903	1.000			
RTDY	0.823	0.850	0.828	0.816	1.000		
RTVA	0.851	0.878	0.858	0.834	0.932	1.000	
RTMI	0.820	0.862	0.841	0.803	0.866	0.907	1.000

Figure 3. Similarity matrix of *R. tetraphylla* generated from Dice estimate of similarity based on the number of shared fragments.

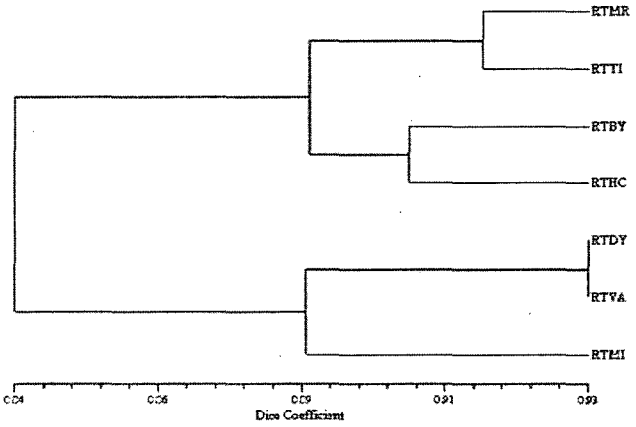

Figure 4. Cluster diagram of seven accessions of *R. tetraphylla* based on Dice genetic identity.

show any effect on amplification. The average number of polymorphic bands per primer generated is 5.1 out of the total number of bands of 9.9. The primers OPA- 01, OPC-02 (Figure 2d), OPC-03 and OPC-17 exhibited 100 % polymorphism. The size of the amplified fragments ranged from 300-3,500 bp.

Levels of genetic diversity within accessions i.e., the genetic distance ranged from 0.816-0.932 (Figure 3). The minimum genetic distance of 0.816 is exhibited between the accessions collected Dulapally and Hyderabad whereas the accessions that exhibited a maximum genetic distance of 0.932 belong to plants collected from Vijayawada and Dulapally. The mean value of genetic distance among the accessions is 2.733. The mean values of genetic distances calculated in accessions which are grouped together in Cluster analysis irrespective of the geographical distances are the accessions collected from Dulapally and Vijayawada showed a genetic distance of 0.932, Balpally and Hyderabad with a genetic distance of 0.903, Mallur and Tirupathi with a genetic distance of 0.915 (Figure 4). The number of accession specific bands (unique markers) are four, which belong to accessions collected from Hyderabad, Balpally, Maredumilli and Mallur.

Discussion

The differences among accessions of *R. tetraphylla*, collected from different locations, could partly be explained as a result of abiotic (geographical, e.g., hydrographic connections, or climactic differentiation. e.g., annual rainfall differences) and biotic (pollination and seed dispersal between *populations etc) factors. It is expected that obligate outcrossing* species show more genetic variation at the population level (Apostol et al., 1996) as observed in *R. tetraphylla*.

With 40 primers polymorphism of 51.6% was revealed among the collected accessions of *R. tetraphylla* and 3 primers among them exhibited 100% polymorphism (Tables 2 ; Figures 2 a-d). The differences found within different accessions suggest that sampling from a few localities for either breeding or conservation could capture large proportion of the variation.

The differences found in the dendrogram generated by RAPDs could be partially explained by different number of PCR products analyzed reinforcing the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships among the plant species of *R. tetraphylla* (Figures 4). Another explanation could be low reproducibility of RAPDs (Karp et al., 1997). The putatively similar bands originating for RAPDs in different accessions of *R. tetraphylla* are not necessarily homologous although they share the same size in base pairs. *This situation may lead to wrong results when calculating* genetic relationships. Problems reliability and repeatability of RAPD markers are well known (Ellsworth et al., 1993). However in our experiments, high reproducibility with PCR products for RAPDs was observed.

Cluster analysis revealed that the accessions of *R.*

tetraphylla collected from Dulapally and Vijayawada show a genetic similarity of 93% though they are quite distant geographically. This situation arises only in the case of natural populations where there is a free/random pollen flow and fertilization, as is the case of the cross-pollinated species. On the other hand the vast genetic variation may serve for its evolution.

Hence the grouping of these accessions is independent of the geographical distance. It proved that the accessions collected from different locations exhibited similarities (leaf morphology and length, colour of the petiole and midrib, flower morphology and fruit morphology) but their RAPD fingerprinting differed markedly.

The gene flow in higher plants is accomplished by dispersal of seeds, pollen as well as by vegetative mobility (Parker and Hamrick, 1992). Gene flow by pollen dispersal is often low in herbaceous plants (Widén and Svenson, 1992). In *R. tetraphylla* none of the accessions collected for our study have less than 50 km distance to each other. Hence, the genetic structure of any of these accessions is stable and free from any gene flow into them. Thus there is a wide range of genetic differentiation. The genetic variation is related to the distances of pollen and seed dispersals.

Results from RAPD analysis indicates that genetic drift might have occurred among the accessions *R. tetraphylla* thereby producing genetic differentiation. The main reason i.e., the human activity that damaged their habitats and their excessive use for medicine make the species population decrease in size and habitat. With a larger area of population, the probability of crossing among the individuals increases, which results in the retention of genetic variation. Though many individuals of *R. tetraphylla* were reported earlier in due course of time they have disappeared gradually along with environmental and edaphic changes in their habitat. For decades much attention has focused on the genetic risks associated with small population size, not only from inbreeding and genetic drift, but also from gene flow. Until now, a precise empirical assessment of how well diversity has been characterized is unavailable (Ellstrand and Elam, 1993).

Apart from genetic drift, inbreeding depression may also be one of the factors, which may lead to genetic variation (Sherwin and Moritz, 2000). An understanding of these genetic processes is required in order to fully evaluate the consequences of fragmentation and its relationship to genetic variation. Inbreeding is avoided in all the accessions of *R. tetraphylla* because the plants are dioecious, although within-population gene exchange between plants is unavoidable.

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 several 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 outcrossing may lead to higher genetic diversity.

It is believed that mutations, genetic drift due to finite population size, and natural selection will lead to the genetic diversification of local populations and that the movement of gametes and individuals (gene flow) will oppose that diversification. The lack of gene flow and the effect of genetic drift due to restricted population size might have caused the accessions of *R. tetraphylla* to differentiate genetically among themselves.

Though *R. tetraphylla* is a common plant, recently it is categorized as endangered due to many environmental and anthropogenic influences (Faisal and Anis, 2002). Palynological and anthropogenic influences may also be attributed to high levels of genetic variation.

The high degree of genetic variation or differentiation recorded that transfer of germplasm between different areas should be avoided, to ensure that the genetic material is adapted to local conditions (Ennos, 1998). The genetic analys presented here could be used for the development of conservation strategies for the species, for example through the definition of appropriate units of management (Newton et al., 1999). Additional information is also required on patterns of variation in quantitative genetic traits, on which plans for conservation action should ideally be based (Ennos, 1998). Due to the unpredictable level of homozygosity and heterozygosity of our accessions the samples were collected at random. Alternatively, if wild populations exist as heterogeneous mixtures of several inbreds, genetic erosion in the gene bank could also occur.

In *R. tetraphylla* substantial heterozygosity may be present which may be lost in due course of time. In studying genetic diversity it is desired to maximize the preservation of alleles. Geographical, climatic or reproductive variables explain the partitioning of the diversity observed which may aid in improving the strategies for maximizing the efficiency of germplasm collection and preservation. Diploidy and self- incompatibility presumably generate high levels of heterozygosity and therefore greater vulnerability to loss of diversity (Loveless and Hamrick, 1989).

In *R. tetraphylla* gene flow homogenizes population structure and counteracts the effects of drift and diversifying selection. It may also be detrimental to small populations because under certain conditions it may reduce local variation, prevent local adaptive differentiation and reduce fitness. Thus populations can undergo genetic differentiation from one generation to another (Slatkin, 1987). Further amplification of such cross-hybridized seeds

through dissemination by natural modes like wind is possible. This is probably the reason that accessions are closely related at the genetic level, although, are geographically from distinct areas of Andhra Pradesh. Marked cyclic fluctuations in population numbers are well documented for *R. tetraphylla* where populations are seen to disappear completely for periods of time only to be replaced by large numbers of vigorous new recruits presumably from buried seed reserves. Extant plants are thus only part of the gene pool and a large genetic reserve lies in buried seed banks, the size and long-term viability of which is unknown. There are many natural stands which are being lost due to many factors, therefore efforts should be made to conserve the remaining stands mostly under *ex situ* conditions.

Unlike two-primer mediated PCR, RAPD assay is performed using low stringency conditions. By interference, mismatches may occur between the primer and its target sequence in the amplification reaction. In fact different thermal cyclers, temperature profiles, the brand of DNA polymerase, and the concentration of $MgCl_2$, primer and template DNA can affect the reproducibility of RAPD assay (Macpherson et al.,
1993). Thus, a standardized methodology devised for RAPD assay to obtain identical RAPD pattern. Similarity can be used to measure the relatedness of samples. Higher levels of genetic variability might be because of strict self incompatibility (Wolff et al., 1994).

Conclusions

Therefore analysis of RAPD data could be useful to detect genetic differentiation between accessions of *R. tetraphylla*. However detailed study is desirable to understand all the aspects related to variations. Hence further information is required on patterns of gene flow within and between accessions, and its effects on reproductive and demographic processes, to assess its impact on population viability.

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