Int. J. Mendel, Vol. 30 (1-4), 23-27, 2013 **BIOCHEMICAL GENETICS** ISSN 0970-9649

ASSESSMENT OF GENETIC RELATIONSHIP AMONG *Rauvolfia serpentina* AND *Rauvolfia tetraphylla* USING RESTRICTION ENDONUCLEASE, RAPD PCR AND MORPHOLOGICAL CHARACTERS

Runa Rashmi* and Maheshwar Prasad Trivedi**

Key words : Assessment, genetic relationship, *Rauvolfia* spp, Restriction Endonuclease, RAPD PCR, morphological characters.

The study was conducted for assessment of genetic relationship between two selected species of *Rauvolfia* using Restriction Endonuclease, RAPD PCR and Morphological characters. 11 primers were used out of which the amplifications of only 5 primers (OP-01, OP-03, OP-05, OP-07 and OP-08) were satisfactory. In Restriction Endonuclease, EcoRI and Hind III gave many bands of agarose gel. This work can lead to development of an efficient protocol to study the genetic relationship between two selected species of *Rauvolfia*.

INTRODUCTION

The genus *Rauvolfia* L. (Apocynaceae) comprises of 80 species distributed in tropics of the world (Mabberley, 2008). India is the largest producer of medicinal herbs and is appropriately called the botanical garden of the world (Ahmedulla and Nayar, 1999). Two species of *Rauvolfia*, viz., *R. serpentina* (L.) Benth. ex Kurz and *R. tetraphylla* L. occur in India (Nayar *et al.*, 2006). *Rauvolfia tetraphylla* is a native of the West Indies but naturalised in India. *Rauvolfia serpentina* is the source of indole alkaloids, including the therapeutically useful compound 'reserpine' for example to treat cardiovascular diseases (Anitha and Kumari, 2006), hypertension (Von Poser *et al.*, 1990), arrhythmia (Kirillova *et al.*, 2001), various psychiatric diseases (Bhatara *et al.*, 1997; Kirtikar and Basu, 1993), breast cancer (Stanford *et al.*, 1986), human promyelocytic leukemia (Itoh *et al.*, 2005) etc. Pharmacological activity of *Rauvolfia tetraphylla* is mainly due to the presence of alkaloids like ajmaline, ajmalicine, reserpine, serpentine and tetraphyllincine. Reserpine is a potent alkaloid that epresses the central nervous system and lowers blood pressure. The root is also used to stimulate uterine contraction and is recommended for use in difficult child birth cases. Exports of these species are banned by the Government of India in order to prevent over exploitation of this species from the wild. *Rauvolfia* is threatened in India due to indiscriminate collection and over exploitation of natural resources for commercial purposes to meet the requirements of pharmaceutical industry, coupled with limited cultivation (Nayar and Sastry, 1987; Gupta, 1989). IUCN has kept this plant under endangered status. Though considerable variation can be observed among *Rauvolfia serpentina* and *Rauvolfia tetraphylla* in gardens around the world, attempts have not been made so far to study the genomic relations between the two. The Polymerase Chain Reaction (PCR) based on molecular markers such as Random Amplified Polymorphic DNA (RAPD) and Restriction Digestion are being extensively used to study the genetic diversity in a number of plant species. To date, no report is available on applications of molecular markers in studies on the genetic diversity of *Rauvolfia serpentina* and *Rauvolfia tetraphylla*. In this context, this work can lead to development of an efficient protocol to study the genetic relationship between two selected species of *Rauvolfia* using Restriction Endonuclease, RAPD PCR and Morphological characters.

MATERIAL AND METHODS

Plant Material

The study involved two species of *Rauvolfia*, viz., *R. serpentina* and *R. tetraphylla* grown naturally in the departmental garden of Botany, Patna University, Patna-5 (Table-1 and Figure-1). Detection of different species was based on the morphology (Shaw *et al.*, 2009). Leaf samples of each species were collected from plants in three locations (populations). Five plants of each species were selected in each location based on morphological homogeneity.

Isolation of Genomic DNA

Genomic DNA was isolated from tender young and fresh leaves by the standard CTAB (Cetyl trimethyl ammonium bromide) method (Doyle and Doyle 1990) with slight modifications. Insoluble polyvinylpolypyrrolidone (PVPP) was added to the leaf tissue prior to grinding. RNA was removed by RNaseA treatment. The DNA concentration was determined by comparison with a known concentration of lambda phase DNA (Sambrook *et al.*, 1989). The DNA quality as well as quantity was also checked by agarose gel and an UV-VIS Spectrophotometer (U.V Mini 1240). After quantification, the DNA was diluted with TE buffer (Tris 10mM and EDTA 1 mM, pH 8.0) to a working concentration of 25 ng mL-1 for PCR analysis.

Random Amplified Polymorphic DNA (RAPD) analysis

The RAPD analysis was performed following the methodology (Williams *et al.*, 1990) with slight variations. Primer used was synthesized by Qiagen Pvt Ltd. Sets of 11 decamer primers were used for amplification of DNA. Conditions were optimized for RAPD-PCR assay (Table-2a. and 2b.) using 50 µl reaction (GeNei™) containing 32.50 µl

Department of Botany, Patna Science College, Patna University, Patna, Bihar-800 005, India.
***Corresponding Author**
E-mail- runa@zhi.org.in, mptrivedi1956@rediffmail.com

autoclaved water, 1.5 µl of each dNTP, 7.5 µl of 10X PCR buffer, 1.5 µl of Taq DNA polymerase, 1 µl of primer and 6 µl of DNA template (extracted DNA from 1 ml of test sample dissolved in 50 µl of TE buffer). PCR amplification was carried out in thermal cycler using the optimized programme (Table- 3).

Restriction Endonuclease Digestion

Restriction Endonuclease Digestion of DNA was done with the help of GeNei™ kit. Two different reaction mixtures in 1.5 ml vials were prepared. Reaction 1 contained 20 µl test DNA, 25 µl 2X assay buffer and 3 µl EcoR I whereas Reaction 2 contained 20 µl test DNA, 25 µl 2X assay buffer and 3 µl Hind III . The vials were incubated at 37^0C for an hour. After an hour 5 µl of Gel Loading Buffer was added to each of vials to stop the digestion.

Agarose Gel Electrophoresis

Genomic DNA (5 µl) and gel loading dye (3 µl) was separated in 0.8% agarose gel (Fig 2).The amplicons were separated in 1.2% and 2 % agarose gel for RAPD (12 µl DNA + 3 µl dye) and Restriction Endonuclease Digestion (12 µl DNA + 3 µl dye) respectively. Electrophoresis was performed at a constant voltage at 60 V for 3 hours. The amplicons were visualized under UV light (Transilluminator Bangalore GeNei™) and photographed (Figs. 3 and 4). The amplicon size was determined by comparison with the ladder (Bangalore GeNei™ Ruler 100 bp ladder plus). The entire process was repeated twice to ensure reproducibility.

RESULTS AND DISCUSSION

In this investigation, 11 random decamer oligonucleotide primers were used for *Rauvolfia serpentina* and *Rauvolfia tetraphylla*. Out of these 11 primers, the amplifications of only 5 primers (OP-01, OP-03, OP-05, OP-07 and OP-08) were satisfactory and reproducible. The reason for the non-amplifications of the other 6 primers could not be explained. Probably the sample DNA did not have any binding site for the primers. A similar nonamplification of decamer primers was reported (Hosaka *et al.*, 1984; Cisneros and Quiros 1995; Sosinski and Douches 1996 and Mattagajasingh *et al.*, 2006), in different plant species. The amplification pattern is shown in Figure 3 (a) and the details of the RAPD analyses (Table 4). All these 05 primers resulted in the amplification of 25 bands, indicating the presence of a high degree of genetic variation in the studied species. All primers obtained a wide range of amplicons, ranging from 640 bp to 1750 bp. The largest amplicon (1750 bp) in this study was amplified by the primers OP-07 and the shortest (640 bp) by OP-03. The highest number of amplicons (9) was observed for primer OP-05 and the lowest (3) for the primers OP-03. In this investigation, RAPD markers were successfully used to differentiate each other. Thus, on the basis of RAPD, the findings of this study are similar to observations (Rajaseger *et al.*, 1997 and 1999). Our results also agree with findings of Loh *et al.*, (1999) who used AFLP markers to study genetic diversity in *Caladium bicolar*.

Different banding patterns of restriction endonuclease digested DNA showed that the DNA sequence of *Rauvolfia serpentina* and *Rauvolfia tetraphylla* is not same.

Thus, RAPD, Restriction Digestion and morphological characters are highly correlated with the combined markers suggesting that all markers are relevant to study the genetic relationships among *Rauvolfia serpentina* and *Rauvolfia tetraphylla*.

ACKNOWLEDGEMENTS

The authors are thankful to the Indian Institute of Bioinformatics and Biotechnology, Patna, India, for providing infrastructure and technical support during experiments.

References

Ahmedulla, M., and Nayar M.P., 1999 : Red data book of Indian plants. Vol-4, Calcutta : Botanical survey of India.

Anitha, S., and Kumari B.D.R., 2006 : Stimulation of reserpine biosynthesis in the callus of *Rauvolfia tetraphyla* L. by precursor feeding. Afr. J. Biotechnol., **5** : 659-661.

Bhatara V.S., Sharma J.N., Gupta S., and Gupta Y.K., 1997 : Rauwolfia *serprentina*: The first antipsychotic. Am. J. Psychiatry, **154** : 894-894.

Cisneros P.L., and Quiros., 1995 : Variation and phylogeny of the triploid cultivated potato olanum chaucha based on RAPD and Isozyme markers. Genetic Research and Crop Evolution **42** : 373-386.

Doyle J.J., and Doyle J.L., 1990 : Isolation of plant DNA from fresh tissue. Focus **12** : 13 -15.

Gupta, R., 1988 : Genetic resources of medicinal plants, Indian Journal of Plant Genetic Resources 1 : 98-102.

Hosaka K., Mori M., and Ogawa K., 1984 : Genetic relationship of Japanese potato cultivars assessed by RAPD analysis. American Potato Journal **71** : 535-546.

Itoh A., Kumashiro, T. Yamaguchi, M., Nagakura N, Mizushina Y, Nishi T., and Tanahashi, T., 2005 : Indole alkaloids and other constituents of *Rauwolfia serpentina*. J. Nat. Prod., **68** : 848-852.

IUCN, WHO and WWF., 1993 : Guidelines on the Conservation of Medicinal Plants. IUCN, Gland, Switzerland, ISBN : 2-8317-0136-8.

Kirillova, N.V., Smirnova M.G., and Komov V.P., 2001 : Sequential isolation of superoxide dismutase and ajmaline from tissue culture of *Rauwolfia serpentina* Benth. Prikl. Biokhim. Mikrobiol., **37** : 181-185.

Kirtikar, K.R., and Basu, B.D., 1993 : Indian Medicinal Plants. Vol. 2, Bishen Singh Mahendra Pal Singh Publishers, Dehradun, India, pp : 289.

Loh, J.P., Kiew, R., Kee, A., Gan, L.H., and Gan, Y.Y., 1999 : Amplified Fragment Length polymorphism (AFLP) provides molecular markers for the identification of Caladium biocolor cultivars. Annals of Botany **84** : 155-161.

Mabberley D.J., 2008 : *Mabberley's Plant-Book : A portable dictionary of plants, their classification and uses.* Third Edition. Cambridge University Press, Cambridge.

Mattagajasingh, I., Mukherjee, A.K., and Das, P., 2006 : Genomic Relations Among 31 Species of Mammillaria Haworth (Cactaceae) Using Random Amplified Polymorphic DNA. Z Naturforsch **61** : 583-591.

Nayar, M.P., and Sastry, A.R.K., 1987 : Red Data Book of Indian Plants. Vol. 1, Botanical Survey of India, Calcutta.

Nayar, T.S., Beegam A.R., Mohanan, N., and Rajkumar, G., 2006 : *Flowering Plants of Kerala.* Tropical Botanic Garden & Research Institute, Thiruvananthapuram.

Rajasegar, G., Tan, H.T.W., Turner, I.M., and Kumar, P.P., 1999 : Random Amplified Polymorphic DNA variation among and within selected Ixora *(Rubiaceae)* populations and mutants. Annals of Botany **84** : 253-257.

Rajaseger, G, Tan, H.T.W., Turner, I.M., and Kumar P.P., 1997 : Analysis of genetic diversity among Ixora cultivars *(Rubiaceae)* using random amplified polymorphic DNA. Annals of Botany **80** : 355-361.

Sambrook, J., Fritsch, E.F., and Maniatis, T., 1989 : Molecular cloning a laboratory manual. 2nd ed., **3** : 18-47.

Shaw, R.K., Acharya, L., Mukherjee, A.K., 2009 : Assessment of genetic diversity in a highly valuable medicinal plant *Catharanthus roseus* using molecular markers. Crop Breeding Appl Biotech **9** : 52-59.

Sosinski, B., and Douches, D.S., 1996 : Use of PCR based DNA amplification to fingerprint North American potato cultivars. Horticulture Science **31** : 130-133.

Stanford, J.L., Martin, E.J., Brintin, L.A., and Hoover, R.N., 1986 : Rauwolfia use and breast cancer : A case-control study. J. Natl. Cancer Inst., **76** : 817-822.

Von, Poser G., Andrade, H.H., Da Silva, K.V., and Henriques, J.A., 1990 : Genotoxic, mutagenic and recombinogenic effects of *Rauwolfia alkaloids.* Mutat. Res., **232** : 37-43.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V., 1990 : DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl Acids Res **18** : 6531-6535.

TABLE -1 : Morphological characteristics of *Rauvolfia serpentina* and *Rauvolfia tetraphylla.*

Sl. No.	Diameter and Place of Flower	Species	Leaf	Flower Smell	Fruit
1.	Flowers are in irregular corymbose cymes, white, often tinged with violet. The inflorescenece with red pedicels and calyx and white corolla.	*Rauvolfia serpentina*	The Leaves of the plant are in whorls of three to five, elliptic to lanceolate or obovate, bright green above and below pale green and thin.	Light sweet scented	Its fruits are tiny, oval, fleshy drupe, single or didymous, shining green which turn a shiny purple -black when ripe.
2.	The flowers grow in clusters at the end of each branch; 2.5-5 cm diameter, with a deeply 5-lobed fringed corolla round the central corolla tube.	*Rauvolfia tetraphylla*		sweet-scented	Its fruits are tiny, circular, fleshy drupe, in groups, shining green which turn a shiny purple-black when ripe.

TABLE-2a : The master mixture for optimization of RAPD PCR (50 µl reaction volume).

NAME	VOLUME
Number of reactions	3
Reaction volume	50 µl
Total volume	150 µl
Autoclaved water	97.50 µl
10X PCR buffer with 15mm $MgCl_2$	22.50 µl
dNTP mixture	4.5 µl
Taq polymerase	4.5 µl
Primer	3 µl
Total volume	132 µl
Each reaction	44 µl master mix. + 6 µl isolated DNA

TABLE-2b: Samples preparation for RAPD PCR for isolated genomic DNA of *Rauvolfia serpentina* and *Rauvolfia tetraphylla.*

SL. NO.	SAMPLE	VOLUME
1	*Rauvolfia serpentina*	44 µl of M.M + 6 µl of isolated DNA
2	*Rauvolfia tetraphylla*	44 µl of M.M + 6 µl of isolated DNA
3	Blank	44 µl of M.M + 6 µl of D.D.Water (Sterilized)
4	Total	132 µl + 18 µl = 150 µl.

TABLE-3: PCR programmed for RAPD (amplification of Isolated Genomic DNA).

Step	Temperature	Time	No. of Cycle
1. Initial denaturation 940C	2 min.	1	
2. A- Denaturation	94°C	30sec.	2
2. B-Annealing	35°C	1 min.	
2. C-Extention	72°C	2 min.	
3. A- Denaturation	94°C	30 sec.	2
3. B- Annealing	35°C	1 min.	
3. C- Extention	72°C	2 min.	
4. A- Denaturation	94°C	30 sec.	43
4. B- Annealing	35°C	1 min.	
4. C- Extention	72°C	2 min.	
5. Final extension	72°C	5 min.	1
6. Hold	4°C	As per convenience	1

TABLE-4 : Details of primers and banding pattern of RAPD analyses in *Rauvolfia serpentina* and *Rauvolfia tetraphylla*

Primer	Nucleotide Sequences	Range of Amplicons (in BP)	Total Bands
OP-01	5'ACGGATCCTG3'	650-1600	5
OP-03	5'AGTCAGCCAC3'	640-1500	3
OP-05	5'GGATCCATATC3'	600-1450	9
OP-07	5'ACGGTCTAAC3'	950-1750	4
OP-08	5'GGGATATCGG3'	800-1650	4

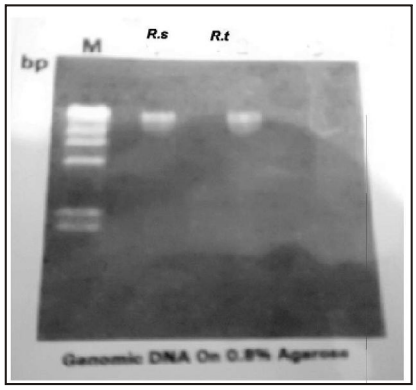
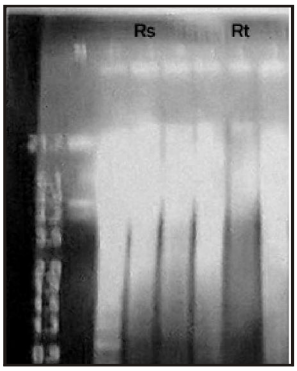


Figure-2: Genomic DNA of *Rauvolfia serpentina* and *Rauvolfia tetraphylla* separated in 0.8% agarose

Figure-3: RAPD PCR product (12 µl amplified DNA + 3 µl gel loading dye) using OP-07 primer along with marker at one side separated in 1.2 % Agarose.



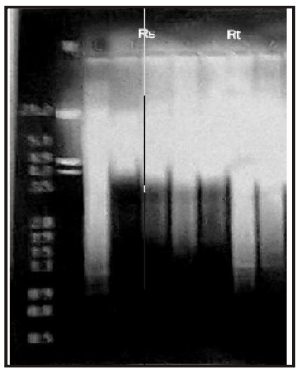


Figure-4: Restriction Endonuclease Digestion product (12 µl amplified DNA + 3 µl gel loading Dye) separated in 2 % agarose (Eco R1 and Hind III respectively)

Figure-1: Morphological characteristics of *Rauvolfia serpentia* and *R. tetraphylla*.