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Research Article

ISSR marker based DNA profiling studies in *Rauwolfia* species

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Abstract: In this present study, a protocol was developed for the DNA profiling of medicinally important *Rauwolfia* species (05 species) by ISSR-PCR method. Initially genomic DNA was isolated from the fresh leaves (1 gram) of 24 months old *Rauwolfia tetraphylla, R.serpentina, R.pentaphylla*, *R.vomitaria* and *R.micrantha*. The content of genomic DNA isolated from 1 gram of fresh leaves of these five species ranges from 1.6 to 3.4 µg. Out of six inter simple sequence repeats (ISSR) primers used, HY3 primer has given the good amplification with distinct bands which were reproducible. The range of DNA bands produced by the HY3 primer were between 300 bp to 1500 bp among the five species. A dendrogram was constructed on the basis of similarity matrix data by unweighted pair group method with arithmetic average (UPGMA) cluster analysis using indigenous software. Average genetic similarity generated by HY3 has revealed 38% similarity between species of Sub cluster-A and species of Sub cluster-B which is consistent with their geographical distribution i.e. Sub cluster-A species *R.tetraphylla* and *R.serpentina* inhabit peninsular India, Sub cluster- B species *R.micrantha* and *R. vomitoria* inhabit the Himalayan foot hills. The developed protocol of DNA profiling in *Rauwolfia* species by ISSR-PCR method could be used efficiently for the differentiation and identification of the *Rauwolfia* species at molecular level.

Key words: Rauwolfia, DNA Profiling, ISSR-PCR, Dendrogram Analysis

Introduction

In the Apocynaceae family among the 250 genera, the genus *Rauwolfia* is commonly called as snake weed which possess more than 100 species and most of them has lot of medicinal importance due to their traditional use in Indian system of Ayurveda. The genus *Rauwolfia* was named in honour of the German physician Dr Leonhard Rauwolf, who studied several plant species of this genus, while travelling in India (Douglas, 2015). *Rauwolfia* genus has gained lot of importance after the Indian physician Vakil introduced it to the Western countries where he used roots of *Rauwolfia serpentina* for the successful treatment of several patients who were suffering with high blood pressure (Vakil, 1949).

Among the different species of *Rauwolfia*, five species (*Rauwolfia serpentina, R.tetraphylla R.petaphylla, R.vomitoria* and *R.micrantha*) were exploited more due to the presence of commercially important alkaloids such as ajmalicine, ajmaline, deserpidine, isoreserpiline, reserpine, reserpine, reserpiline, raujemidine, serpentine, tetraphylline, sarpagine, vellosimine and yohimbines (Mukherjee, 1961). It is well known that *R.serpentina* is native to India (Vakil, 1949), while all the remaining four species are exotic flora, which are now naturalized in most parts of India (Panda *et al.,* 2012). These five species of snake root plants are now found growing in Indian States like: Andhra Pradesh, Telangana, Tamil Nadu, Kerala, Karnataka, Maharastra, Orissa, Chhattisgarh, Madhya Pradesh, and West Bengal (Singh *et al.,* 1990).

There are several reports about the in vitro micropropagation (Sudha *et al.,*1996; Faisal *et al.,*2012; Rohela *et al.,*2015; Sonibare and Akpan, 2017), secondary metabolites (Shetty *et al.,* 2014; Srivastava *et al.,* 2016), phytochemical screening (Sarika *et al.,* 2012; Rohela *et al.,* 2016), endophytic organisms (Nath *et al.,* 2015), hairy root cultures (Madhusudanan *et al.,* 2008; Menrotra *et al.,* 2013 & 2015); quantitative determination of indole alkaloids (Srivastava *et al.,* 2006); antimicrobial activities (Sarika *et al.,* 2012; Rohela *et al.,* 2015) and pharmacological activities (Bilal *et al.,* 2012; Ezeigbo *et al.,* 2012) from this five species. But there is scanty information available on studying the variability among these species.

Variability among these species can be studied at morphological level like plant height, type of leaf, flower colour, fruit type and seed characteristics

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similar at biochemical level type and quantity of phytoconstituents are routinely used to distinguish the Rauwolfia species. However, these methods were not suitable for the accurate identification of a species, as many discrepancies like locality of plant, influence of environment on trait phenotype, gene interactions will act in changing the characteristic of a species at morphological and biochemical level.

In the modern era of technology, genetic diversity among the related species of a genus can be assessed accurately at molecular level by carrying out DNA profiling using molecular markers like SSR, ISSR, SNP's, RAPD and AFLP which is reported in several genera like Vicia (Megahed *et al.,* 2015), Solanum (Hong *et al.,* 2014), Rheum (Wang *et al.,* 2012), Capsicum (Prasad *et al.,* 2013), Luffa (Yamuna Prasad *et al.,* 2017; Sujatha *et al.,* 2013) etc. These molecular markers are more efficient than phenotypic and biochemical markers and the technique is simple, low cost and does not use radioactive probes (Sitthiwong *et al.,* 2005).

To the best of our knowledge, until now as there are no reports on genetic diversity studies in *Rauwolfia* species, now through this research study we are reporting for the first time about the Genetic diversity among the *Rauwolfia* species through the ISSR-PCR method based method of DNA profiling.

Materials and Methods

Plant Material: *Rauwolfia* species *viz. Rauwolfia tetraphylla, R.serpentina, R.pentaphylla*, *R.vomitaria* and *R.micrantha* were procured from Forest Department, Warangal, Telangana, India in January, 2009. They were maintained in the medicinal arboretum of the Department of Biotechnology, Kakatiya University, Warangal, Telangana and were used in the present investigation.

Isolation of plant genomic DNA using C-TAB method: Genomic DNA was isolated fromfresh leaves of 24 months old *Rauwolfia tetraphylla, R.serpentina, R.pentaphylla*, *R.vomitaria* and *R.micrantha* by following the protocol of Doyle and Doyle, (1990). The method is as follows.

a. Fresh leaves (1 gram) of five species of *Rauwolfia* was grounded into a fine paste using 500µl of C-TAB buffer.
b. Leaf extract was transferred to eppendorf tubes and incubated for about 15 minutes at 55°C in a dry bath.
c. After incubation, the leaf extract was centrifuged at 12000 rpm for 5 min, to pellet out cell debris and to the supernatant 250 µl of Chloroform: Iso-amyl alcohol (24:1) was added and mixed by repeatedly inverting the tube.
d. After proper mixing, the eppendorf tubes were centrifuged at 13000 rpm for 1 min.
e. Upper aqueous layer (containing DNA) was transferred to a clean eppendorf tube, to which 50 µl of 7.5M ammonium acetate and 500 µl of ice-cold absolute ethanol/ iso propanol were added and inverted slowly for several times to precipitate the isolated DNA.
f. To further purify, the precipitated DNA was washed with 500 µl of ice-cold 70 % ethanol for several times.
g. Final centrifugation was carried at 13000 rpm for 1 minute, supernatant was removed and the DNA pellet was air dried.
h. The DNA pellet was dissolved in 1X TE buffer and DNA quantification was performed using Spectrophotometer to determine the concentration and purity of DNA samples.
i. Pure DNA of 20 µl (25 ng) was used in PCR experiment.

Quantification and Quality analysis of isolated DNA

To quantify the amount of DNA isolated from the 1 Gram of Leaf sample of 5 *Rauwolfia* species, we have used the spectrophotometer measurements of the amount of ultraviolet irradiation absorbed by the bases at 260 and 280 nm (Korra *et al.,* 2017).

DNA quality checks

DNA quality check was made through a test gel run to know whether the isolated DNA is in degraded or undegraded state. Intact, undigested DNA forms a band, where as a smear indicates degradation (longer the smear, more is the extent of degradation). Slight degradation sometimes may not be detected, while a highly degraded DNA may appears as a diffuse spots near the end of the gel.

ISSR-PCR Analysis

ISSR analysis was performed as per the method of William's *et al.,* (1990) by using ISSR primers (Table-1). PCR mixture was prepared using the following quantities of chemicals given in Table-2 and the amplification was carried out in a thermocycler (Gene Amp PCR system 9700, Applied Biosystems, California, USA), the programming conditions are given in Table-3.

To reduce the possibility of cross contamination in amplification reaction, a master reaction mixture was routinely prepared and a negative control was also be used; this control consisted of PCR reaction mixture excluding DNA template. Amplification was performed at least twice and only reproducible amplified PCR products were separated on 1.5% Agarose gel.

Agarose Gel Electrophoresis (AGE)

The Agarose gel electrophoresis was performed by the following steps.

a. Agarose was dissolved in 1X TAE buffer by heating in microwave oven.

b. Molted Agarose was cooled to 55° C and 5 µl of Ethidium bromide was added to it.
c. Forty (40) ml of Agarose solution was poured into a gel tray and a comb was inserted at one end and left for gel formation.
d. The comb was removed from the gel to form wells.
e. Agarose gel tray was inserted into Electrophoretic unit containing 1X buffer.
f. One tenth (1/10) volume of dye was added to 10 µl DNA sample.
g. In the first well 10 µl of molecular marker (0.5-10 Kb) was added and 10 µl DNA sample was loaded into other wells, using micropipette.
h. The Electrophoretic unit was connected to the power supply and the gel was run for 45 minutes with 80 volts of current
i. Finally the power supply was terminated and the gel tray was removed from the unit and viewed under gel documentation system.

Dendrogram Analysis

A Dendrogram was constructed on the basis of similarity matrix data by unweighted pair group method with arithmetic average (UPGMA) cluster analysis using indigenous software by the method described by Sitthiwong *et al.,* 2005. Data generated in ISSR analysis by the method of Nei *et al.,* 1979, which excludes negative data on this equation. Similarity = 2 N_{ab} /(N_a+N_b), where N_a = number of scored amplified fragments in genotype a and N_b = number of scored amplified fragments in genotype b and N_{ab} = number of scored amplified fragments with the same molecular weight shared between genotypes A and B.

Results and Discussion

The amount of genomic DNA isolated from the leaves of 24 months old *Rauwolfia* species *viz. R.tetraphylla, R.serpentina, R.pentaphylla, R.micrantha* and *R.vomitoria* is presented in Fig-1.The content of genomic DNA in these five species ranges from 1.6 to 3.4 µg in 1 gram of fresh leaves which was estimated by spectrophotometer.

The number of bands produced by the HY3 primer ranged between 300 bp to 1500 bp (Table-4). *Rauwolfia tetraphylla*, *R.pentaphylla* & *R.vomitoria* produced 5 bands, while *R.serpentina* produced 4 bands and *R.micrantha* produced 3 bands (Fig-2). A DNA band admeasuring 0.7 kb was observed in all 5 species and DNA band admeasuring 0.9 kb was observed in 4 species.

Dendrogram analysis showed that 5 species of *Rauwolfia* are grouped into two main clusters; Cluster-A and Cluster-B (Fig-3). Cluster-A has single species *R.pentaphylla* and Cluster-B includes *R.tetraphylla*, *R.serpentina*, *R.micrantha* and *R.vomitoria*. Cluster-B has two Sub clusters, Sub cluster-A and Sub cluster-B. Sub cluster-A comprises of *R.tetraphylla* & *R.serpentina* and Sub cluster-B comprises of *R.micrantha* & *R. vomitoria.*

The genomic DNA isolated from *R.tetraphylla, R.serpentina, R.pentaphylla, R.micrantha* and *R.vomitoria* ranged between 1.6 to 3.4 µg. Gurudeeban *et al.,* (2011), also isolated 1.5-2.5 µg of DNA from *Suaeda* sp of Apocynaceae family. The ISSR genetic profile obtained through amplification of genomic DNA of five *Rauwolfia* species was polymorphic. Mahesh *et al.,* (2008), using RAPD markers analyzed *Rauwolfia tetraphylla* plants collected from five locations of thiruvinelli hills in Tamil Nadu, India and reported that there is distinct genetic variability. Similar studies were undertaken in *Arachis hypogea* (Raina *et al.,* 2001); *Morus alba* (Srivastava *et al.,* 2004); *Capsicum annuum* (Sitthiwong *et al.,* 2005); *Chimonanthus praecox* (Zhao *et al.,* 2007); *Catharanthus roseus* (Pietrosiuk *et al.,* 2007); *Dioscorea opposite* (Zhou *et al.,* 2008); *Hyptis* (Kranthi *et al.,* 2011); *Artemisia capillaries* (Shafie *et al.,* 2011); *Rheum officinale* (Wang *et al.,* 2012); *Bacopa monnieri* (Tripati *et al.,* 2012) and Ocimum sp (Sanjay *et al.,* 2012).

RAPD and ISSR markers are very simple, rapid and reliable; they require a small quantity of DNA sample and knowledge regarding DNA sequence to design primers is not necessary. They do not use radioactive probes as in RFLP; hence they are suitable for assessment of genetic fidelity of *in vitro* raised clones.

Among the different *Rauwolfia* species, RAPD and ISSR markers were used for confirming genetic fidelity of tissue culture plantlets of *R.tetraphylla* and *R.serpentina* (Spiridonova *et al.,* 2008; Goel *et al.,* 2009; Tiwari *et al.,* 2011; Faisal *et al.,* 2012 a, 2012 b; Alatar *et al.,* 2012; Rohela *et al.,* 2013). Mehrotra *et al.,* (2012), has reported about genetic fidelity of tissue culture plantlets of *Rauwolfia vomitoria* by using ISSR and RAPD markers.

Except for confirming the genetic fidelity of *in vitro* regenerated plantlets of *R.serpentina*, *R.tetraphylla* and *R.micrantha*, there is scanty information on the application of molecular markers for studying genetic variability of *Rauwolfia* species growing in wild (*in vivo*).

Average genetic similarity generated by HY3 has revealed 38% similarity between species of Sub cluster-A and species of Sub cluster-B which is consistent with their geographical distribution i.e. Sub cluster-A species *R.tetraphylla* and *R.serpentina* inhabit peninsular India (Anitha and Kumari, 2007), Sub cluster- B species *R.micrantha* and *R. vomitoria* inhabit the Himalayan foot hills (Prajapati *et al.,* 2003; Heber *et al.,* 2006). India. It is well known that *R.serpentina* is native to India (Vakil 1949), while all the remaining four species are exotic flora, which are now naturalized in most parts of India (Panda *et al.,* 2012).

Table 1. List of ISSR primers

S.NO	ISSR-Primers
1	HY1: 5'- TCTCTCTCTCTCTCTCTC - 3'
2	HY2: 5'- GAGAGAGAGAGAGAGAC - 3'
3	HY3: 5´-AGAGAGAGAGAGAGAGG - 3´
4	HY4: 5'- GAGAGAGAGAGAGAGAT- 3'
5	HY5: 5'- CGACGACGACGACGACGA- 3'
6	HY6: 5'- TGCTGCTGCTGCTGCTGCTGC- 3'

Table 2. PCR Mixture

S.No	Chemical	Quantity
1	25 ng of genomic DNA	2.0 µl
2	2.5mm dNTPs	2.0 µl
3	Taq buffer(100 mM Tris hydrogen chloride , pH 8.3, 500 mM Potassium chloride and 0.1% gelatin)	2.0 µl
4	25mM Magnesium chloride	2.0 µl
5	ISSR primer (Bioserve)	3.0 µl
6	0.5 unit of Taq DNA polymerase	1.0 µl
7	Autoclaved Milli Q water (PCR-grade water)	8.0 µl
	Total	**20.0 µl**

Table 3. Thermocycler programming conditions used in the study

Steps	Temp (°C)	Duration (min.)	Cycles (No.)
Initial denaturation	94	5.0	1.0
Denaturation	94	1.0	35.0
Annealing	50	1.0	35.0
Extension	72	2.0	35.0
Final extension	72	10.0	1.0

Table 4. Number and size of DNA bands (kb) generated during HY3-ISSR based PCR analysis in five species of Rauwolfia

S.No	Rauwolfia species	Number of bands	Size of bands (Kb)
1	*R.tetraphylla*	5	0.4, 0.5, 0.6, 0.7, 0.9
2	*R.serpentina*	4	0.5, 0.6, 0.7, 0.9
3	*R.pentaphylla*	5	0.6, 0.7, 0.9,1.5, 2.2
4	*R.micrantha*	3	0.4, 0.5, 0.7
5	*R.vomitoria*	5	0.3, 0.4, 0.7, 0.9,1.3

Figure 1. Quantity of genomic DNA isolated from 1-gram leaf tissue of 24 months old Rauwolfia plants (5 species)

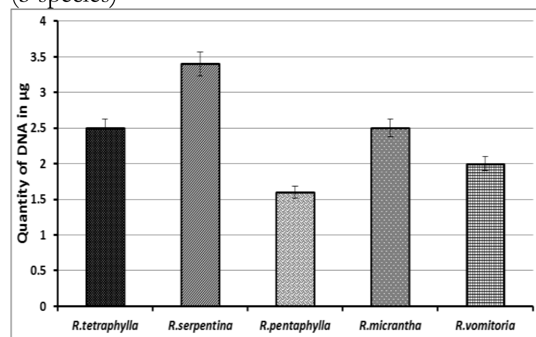
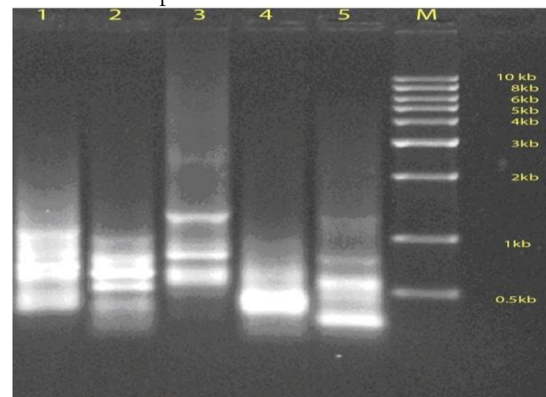
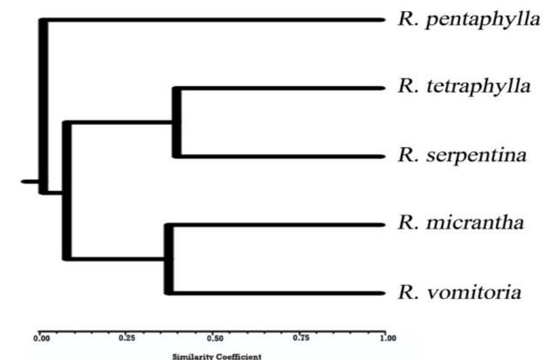


Figure 2. HY3-ISSR primer based DNA profiling in Rauwolfia species



Lane 1 : *R.tetraphylla* ; **Lane 2 :** *R.serpentina* ; **Lane 3 :** *R.pentaphylla* ; **Lane 4 :** *R.micrantha*; **Lane 5 :** *R.vomitoria*; **Lane M :** DNA marker

Figure 3. UPGMA dendrogram of 05 Rauwolfia species based on Jaccard similarity coefficients converted from HY3-ISSR marker data.



Conclusion

Among the different ISSR primers used, HY3-ISSR has generated reproducible polymorphism with distinct DNA bands among the five *Rauwolfia* species which has enabled their identification at molecular level. The developed protocol of DNA profiling in Rauwolfia species by HY3-ISSR-PCR method could be used efficiently by the researchers for the differentiation and identification of 5 mentioned species at molecular level.

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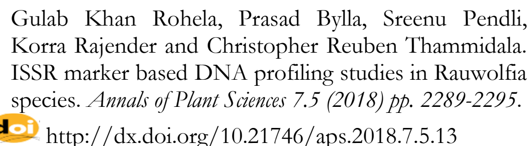

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