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Evaluation of Genetic Variability Among *Dipteracanthus patulus* (jacq) Nees. Assessions using RAPD Markers

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Abstract

Evaluation of genetic variability represents the eco-geographical survey of molecular variation of important medicinal herb *Dipteracanthus patulus* (jacq) Nees. (Haadjud) It is used in treatment of bone fracture. Despite of its diverse medicinal properties no molecular data is available about the pattern of variation in its natural range. In this study, 9 RAPD markers were used to detect genetic variations of 10 accessions collected from 8 districts of Haryana and Rajasthan of India. A total of 65 bands were scored corresponding to an average of 7.22 bands per primer with 41 bands showing polymorphism (63.07%). Six out of nine primers gave more than 60% polymorphism. Jaccard similarity coefficient ranged from 0.58 to 0.93. The similarity coefficient matrix generated was subjected to UPGMA to generate clusters using NTSYS 2.02 pc program. The dendrogram has put all the genotypes in two major groups A and B at 66.25% between group similarities. Group A consists 5 genotype having 79.0% within group similarity and group B 5 genotype with 73.5% within group similarity. This study revealed rich genetic diversity among *Dipteracanthus patulus* accessions from Haryana and Rajasthan.
Keywords: Dipteracanthus patulus; molecular marker; RAPD; Genetic variability

Introduction

Germplasm characterization is an important link between the conservation and utilization of plant genetic resources. The ability of a species to respond adaptively to environmental changes depends on its genetic variability (Ayala *et al*., 1984). The amount and distribution of variation among and within populations result from dynamic processes such as gene flow, selection, inbreeding, genetic drift, and mutation (Hartl *et al.*, 1997). A species without enough genetic diversity is thought to be unable to survive in a changing environment or protect itself against evolving competitors and parasites. Therefore, investigations of genetic diversity and the genetic structure of populations within a species may not only illustrate the evolutionary process and mechanism, but also provide useful information for

biological conservation and phylogenetic analysis (Schaal *et al.*, 1991). Genetic diversity and structure of a species cannot always be predicted on the basis of distribution size and life history and that, therefore, they must be independently studied for each species.

Dipteracanthus patulus (Jacq) Nees (Acanthaceae) is an herbaceous, annual or perennial species. In India it is distributed in Tamil Nadu, Western Ghat, Andhra Pradesh, Rajasthan and Haryana. It is commonly known as Kayappacchilai in Tamil Nadu, Chilanthippacha in Western Ghat and Haadjud in Haryana. In South India it is found on wastelands but in North-west India it is reported only on rocky soil of Aravli hills but not found on plain wastelands. Beside this, it is found as an annual plant in North-west India including Haryana but as a perennial plant in South India (Manikandan *et al*., 2009). It is well known for its pharmacological properties. It showed the wonderful anti- oxidant, antiulcer (Manikandan *et al*., 2009), cardiotonic (Akthar *et al.*, 1992), antibacterial (Kadyan *et al.*, 2010) and wound healing (Saroja *et al.*, 2009) properties. Beside these it is well known for a variety of purposes in indigenous and folk medicines including insect bite, paranychia, venereal diseases, rheumatic complaints, syphilis, gonorrhea, renal infections, eye diseases and bone fracture (Saroja *et al.*, 2009).

Traditionally in Haryana and Rajasthan decoction of stem with cow milk is taken orally for the treatment of bone fracture, and paste of stem with mustered oil is applied topically to relieve muscle pain. Extract of whole plant with sugar is used for the treatment of pneumonia in children's (Yadav *et al.*, 2009). This plant differs a lot in morphological features collected from different regions of India according to climate, soil type and other factors. This plant is an annual herb in Haryana (Northern India) but a perennial herb in Tamilnadu (Southern India) (Saroja *et al.*, 2009). Similarly, due to seasonal and geographical variations, a lot of phytochemical differences were also observed on plants in different studies (Yadav *et al*., 2010; Arya *et al*., 2010).

Phytochemical and morphological differences are directly or indirectly linked to the genetic diversity. Genetic diversity is measured through various molecular markers in which RAPD is most suitable for preliminary analysis because no need to prior information on the genome. RAPD is convenient to operate, with good polymorphism can be used in analyzing genetic diversity and the relation between species has been used in analyzing the relationships between strains belonging to same genera and genetic diversity on many plants (Lanying *et al.*, 2009). Although RAPD is of dominant nature, several strategies have been put forward to minimize the dominance effects on genetic variation analysis (Lynch *et al.*, 1994; Stewart *et al.*, 1996). In occasional cases, RAPD is poor in reproducibility but this can usually be solved by optimization of reaction conditions (Szmidt *et al.*, 1996; Weising *et al.*, 1995). RAPD analysis provides information that can help to define the distinctiveness of species and phylogenetic relationships at molecular level. In present study, the genetic diversity of *Dipteracanthus patulus* populations from different eco-geographic regions has been investigated using RAPD markers and evaluating their overall suitability in such studies.

Materials and Methods

Plant material

To investigate the genetic diversity within species of *Dipteracanthus patulus* (Jacq) Nees leaf samples were collected from ten different geographical populations representing two States (Haryana and Rajasthan) and eight districts of India. Random sampling was done to collect ten accessions for DNA isolation from ten different locations *i.e.* Rewari, Manesar, Behror, Jaipur, Dosi, Alwar, Dadri, Mahindergarh, Patan and Jhunjhunu (Figure 1). Leaf samples from mature plants of approximately same age (based on size, height etc.) were selected for collection. The samples were rinsed with 70% ethanol, air dried, packed in plastic bags and stored in ice box to transfer to laboratory for DNA Extraction.

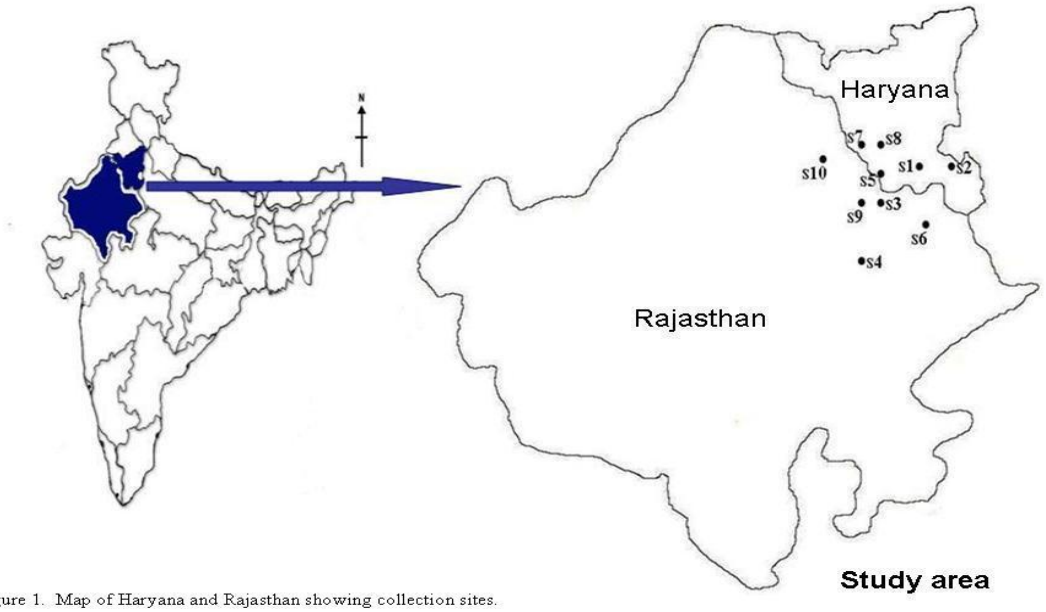

Figure 1. Map of Haryana and Rajasthan showing collection sites.

(S1= Rewari, S2= Manesar, S3= Behror, S4= Jaipur, S5= Dosi, S6= Alwar, S7= Dadri, S8= Mahindergarh, S9= Patan and S10= Jhunjhunu).

DNA Extraction

Genomic DNA was isolated from leaves using Promega Maxwell DNA extractor (USA) and treated with RNase to eliminate RNA. DNA concentration was measured by using Nanodrop spectrophotometer (ND-1000, version 3.1.1, USA). The integrity of the isolated DNA was verified by visualization of DNA on 0.8% Agarose gel with DNA standard uncut lambda DNA. A single sharp band was observed for isolated DNA corresponding in size to λ DNA. The quality of DNA was determined as the ratio A_{260}/ A_{280}, which ranged from 1.8 to 1.9, which is indicative of good quality plant DNA. DNA was diluted to 25 ng/µl for PCR amplification.

Primers

The primers used for amplification were purchased from UBC (University of British Columbia). Sixty random primers (UBC 101-140, and UBC 181-200 from

RAPD set # 2) were screened using Polymerase Chain Reaction (PCR). Nine primers (Table 1) showing good polymorphic bands were used for UPGMA analysis.

RAPD PCR

Sixty random decamer RAPD primers were screened which were procured from UBC (University of British Columbia). The GC contents of primers varies from 50% to 80% were used for RAPD analysis. The RAPD analysis on each primer was repeated twice and if needed thrice to check the reproducibility. The amplification reactions were performed in a final volume of 25 µl containing genomic DNA 25 ng, primer 0.2 µM (UBC), Taq DNA polymerase 0.5 U (Banglore Genei Pvt. Ltd., India), dNTPs 2.5 mM (Banglore Genei Pvt. Ltd., India), and 1x Taq polymerase buffer. Amplifications were done in a My Cycler thermal cycler (Biorad, USA) employing the following conditions: Initial denaturation at 94^0C for 4 min, 44 cycles at 94^0C for 1 min, 37^0C for 1 min and 72^0C for 2 min, with a final extension at 72^0C for 7 minutes. After completion of the cycle, PCR products were stored at –20 ºC until further use. Amplification products were separated on 1.8 % agarose (Sigma-Aldrich, USA) gel in 1X TAE buffer along with a DNA ladder (Fermentas). The DNA was stained by adding ethidium bromide 2 µl (50mg/ µl stock) into the gel mixture, for better visualization. The DNA bands were photographed under ultra violet light using the gel documentation system (Alpha Innotech, USA).

Data analysis

The reproducible bands were scored as present (1) or absent (0) across all accessions. Each band was regarded as locus. All calculations were done using computer program NTSYS pc version 2.02 (Rohlf *et al*., 1998). Similarity matrix was constructed using the Jaccard's similarity coefficients and subjected to UPGMA (unweighted pair-group method with arithmetic averages) analysis to generate dendrogram (Figure 3). Polymorphism information content (PIC) was also calculated according to Anderson and his co-workers (Anderson *et al.,* 1993).

Results and Discussion

Significant variations were observed for various characteristics studied among *Dipteracanthus patulus* accessions. This plant differs a lot in morphological features collected from different regions of India according to climate, soil type and other factors. Beside this, it is found as an annual herb in North-west India including Haryana but as a perennial plant in South India (Manikandan *et al*., 2009). In South India it is found on wastelands but in North-west India it is reported only on rocky soil of Aravli hills but not found on plain wastelands. Leaves width, size and color were slightly differing in accessions collected from Dadri (Haryana) and Jaipur (Rajasthan). The leaves were light green and slightly leathery in accessions collected from Dadri while dark green and simple in Jaipur accessions

According to our investigation glycosides and fats are absent in the plant samples collected from rocky soil of Haryana as compared to Tamilnadu, while diterpenes are present in huge amount. These antagonistic results of these findings may be attributed to different geographical locations and climatic conditions for the growth of the plant. Phytochemical and morphological differences directly or

indirectly linked to the genetic diversity. Genetic diversity is measured through various molecular markers in which RAPD is most suitable for preliminary analysis because it is easy and no need to prior information on the genome of the plant. The RAPD technique had been successfully used in a variety of taxonomic and genetic diversity studies (Vyas *et al.,* 2009; Ahmad *et al.,* 2010). In the present investigation RAPD marker system being employed to assess the genetic diversity of *Dipteracanthus patulus* germplasm was quite informative and was able to generate adequate polymorphism and unique DNA fingerprints in ten populations of *D. patulus* collected from different places covering eight districts of Haryana and Rajasthan (Figure 2).

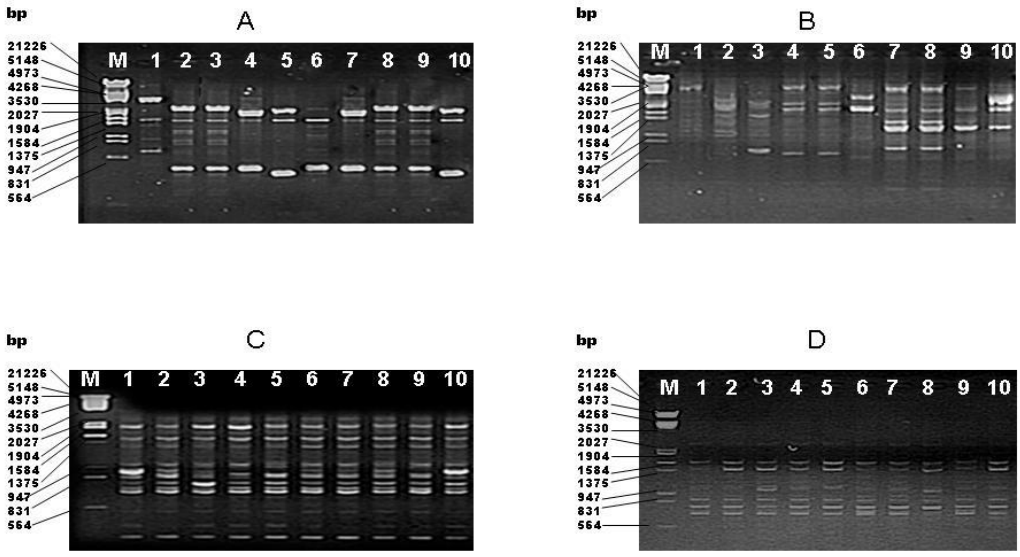

Figure 2. RAPD analysis carried out with Primer: UBC 196 (A), UBC 193 (B), UBC 132 (C) and UBC 184 (D) on Genomic DNA isolated from leaves of *Dipteracanthus patulus*.

Overall, 60 random primers were used to examine the genetic diversity pattern. Of the 60 primers, 15 primers (25 %) resulted in amplification, of which 9 primers gave reproducible and scorable results. Only the bands showing reproducible amplification were considered for scoring and for further analysis.The total number of bands generated by nine amplifying primers were 65 with an average amplification of 7.22 bands per primer. The number of amplification products produced by the 9 primers ranged from 3(UBC 106) to 14 (UBC 132). The number of polymorphic bands ranged from 1 to 12 with range of polymorphisms 16.66% (UBC 184) to 100% (UBC 193) (Figure-2) (Table-1). The average polymorphism generated by these bands was 63.07%. The size of the amplicons generated varied from 272 bp to 3615 bp.

Table 1. List of single arbitrary primers showing total and polymorphic amplicons generated along with PIC values for 10 genotypes of *Dipteracanthus patulus*

Primers	**Sequences** (5'→3')	Total No. of bands (a)	Total No. of polymor-phic bands (b)	Polymor-phism (b/ax 100)	PIC
UBC 106	CGTCTGCCCG	3	1	33.33	0.106
UBC 116	TACGATGACG	5	3	60	0.184
UBC 126	CTTTCGTGCT	5	5	100	0.312
UBC 132	AGGGATCTCC	14	4	28.57	0.084
UBC 137	GGTCTCTCCC	7	6	85.71	0.322
UBC 184	CAAACGGCAC	6	1	16.66	0.070
UBC 189	TGCTAGCCTC	8	5	62.50	0.197
UBC 193	TGCTGGCTTT	4	4	100	0.425
UBC 196	CTCCTCCCCC	13	12	92.30	0.392
Total		**65**	**41**	**63.07 (Aver.)**	

PIC estimates the degree of polymorphism of marker, which essentially is the proportion of individual that are heterozygous for a marker. PIC is a good measure of the heterozygosity. It is an index of how many alleles a certain marker has and how these allele divide. High PIC value indicates rich amount of heterozygosity which in turn associated with a high degree of polymorphism (Bouchard *et al.,* 1997; Zimmer *et al.,* 2005). In the present study, PIC values were ranges from 0.070 (UBC 184) to 0.425 (UBC 193) (Table 1).

Genetic similarity estimates based on RAPD banding patterns were calculated using method of Jaccard's similarity coefficient analysis (Table 2). The similarity coefficient matrix generated was subjected to algorithm "Unweighted Pair Group Method for Arithmetic Average (UPGMA)" to generate clusters using NTSYS 2.02 pc program. The dendrogram showing relationship among various genotypes was constructed using these clusters (Figure 3). The Jaccard's pairwise similarity coefficient values ranged from 0.58 (S 4 and S 2, S5 and S2) to 0.93 (S7 and S4) with an average of 0.711, for single primer based RAPD patterns (Table 2). The clusters constructed through NTSYS (2.02 pc) presented in the form of dendrogram has been shown in Figure -3. The dendrogram has put all the genotypes in two major groups (group A and B) at 66.25% between group similarities. Group A consist S1, S4, S5, S7 and S10 total 5 genotype having 79.0% within group similarity and group B consists S2, S8, S3, S6 and S9, total 5 genotype with 73.5% within group similarity.

Table 2. Jaccard's similarity coefficient among different accessions of *D. patulus*.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1	1.00									
S2	0.65	1.00								
S3	0.62	0.67	1.00							
S4	0.76	**0.58**	0.65	1.00						
S5	0.76	**0.58**	0.65	0.79	1.00					
S6	0.77	0.75	0.79	0.76	0.76	1.00				
S7	0.82	0.60	0.67	**0.93**	0.81	0.78	1.00			
S8	0.64	0.76	0.73	0.67	0.67	0.75	0.69	1.00		
S9	0.65	0.70	0.74	0.68	0.71	0.69	0.70	0.73	1.00	
S10	0.72	0.61	0.65	0.75	0.79	0.76	0.77	0.67	0.62	1.00

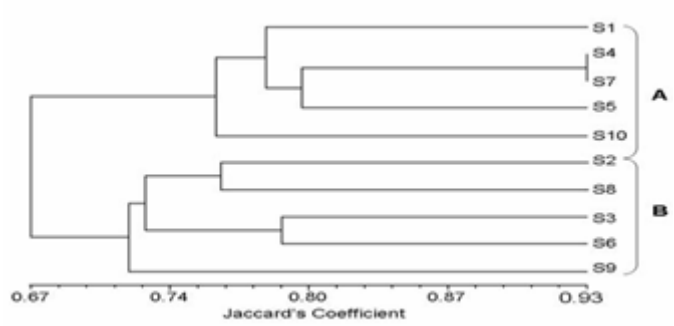

Figure 3 Dendogram showing relationship among ten *Dipteracanthus genotypes* generated by UPGMA analysis based on single primers

Conclusion

Based on polymorphic feature, genetic diversity and genetic similarity among the populations of *Dipteracanthus patulus* based on RAPD study, we conclude that this marker proved an efficient tool in assessing the genetic diversity of *Dipteracanthus patulus* genotypes. The observations and interpretations of this investigation are interesting as a preliminary exploration analysis. The present findings make a strong point to enlarge the scope and size of collection throughout the distribution area of *Dipteracanthus patulus* in order to detect and quantify the prevalent genetic diversity existing within this species at molecular level. Data generated from the present study as well as from similar studies in future would go a long way in conserving distinct genotypes of this species. To the best of our knowledge this is the first report on the characterization of *Dipteracanthus patulus* genotypes based on commercially available primers from Haryana and Rajasthan region. The powerful capability of molecular technique to distinguish closely related genotypes based on their RAPD patterns has been brought out by this study.

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***Authors' contributions**: **Dr. J.P.Yadav** (Professor & Head), Project leader, contributed in experiment design, final editing of the manuscript; and corresponding author of manuscript; **Mr. Sanjay Yadav** (Research student), contributed in experiment design, preparation, wrote and performed some of the experiment; **Dr. Vedpriya Arya** (Researcher), contributed in some of the experimental work and statistical analysis; **Mr.Pawan Kumar** (Research student), contributed in collection of plant material.*

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