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Intraspecific morphological and genetic variations within the populations of *Calotropis gigantea* (L) W.T. Aiton from Shivalik hills of Himachal Pradesh, India

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Abstract

Calotropis gigantea is an important medicinal, drought resistant wild plant of the Himalayas. Present study was aimed to elucidate the intraspecific morphological and genetic variations among the population of *C. gigantea* in Shivalik hills of Himachal Pradesh, India through morphological and RAPD markers, respectively. The samples of the plant were collected in triplicates (each sample was approximately three km far away from each other) from five districts (Sirmour, Hamirpur, Solan, Una and Kangra) of Himachal Pradesh using purposive sampling method. Different qualitative characters studied were habit, external shape of tree, stem, leaf characters, flower characters (calyx, corolla, and roecium, and gynoecium), fruit and seed. On the basis of above qualitative characters, no variation was observed in all the districts except Hamirpur which could be the results of epigenetic change. Additionally, intraspecific genetic variations of five genotypes were analyzed by RAPD technique using five random operon primers. Five primers have generated a total of 36 bands out of which 28 were polymorphic. Cluster analysis was carried out based on JACCARD's similarity coefficient and the UPGMA clustering method which showed 77% polymorphism among the genotypes. Results revealed a high level of genetic variations between the selected variants.

Key words : *Calotropis gigantea*, Intraspecific genetic diversity, Morphological variations, RAPD, UPGMA

INTRODUCTION

Genetic diversity among organisms is an evolutionary character which provides raw material for natural selection. It may be due to the differences in genes and genotypes within and between the populations of organisms. In the past, qualitative characteristics such as leaf shape, leaf colour, flower colour and fruit colour have been used to study genetic variation in combination with quantitative characters like plant height, leaf number, size, etc. But, such morphological traits, also called as descriptors are limited in number. Therefore, single study of morphological characters is not sufficient for the estimation of interspecific or intraspecific genetic diversity in plants. For the last four decades, molecular markers have shown a significant advantage in the analysis of genetic diversity. The molecular markers when used with morphological details, they are more useful and accurate for determination of both intra and interspecific variation in plants and therefore, overcome the limitations of morphological markers. These are Restriction fragment length polymorphism (RFLP), Random amplified polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP), Simple sequence repeat (SSR), etc[1-2]. Among these, RAPD is commonly used marker to observe intraspecific genetic diversity in most of the plant species viz., *Phoneix dactylifera*, *Jatropha curcas*, *Glycyrrhiza glabra*, *Kaemferia galangal*, *Pinus roxburghii*, etc.[3-7].

Calotropis gigantea (L) W.T. Aiton, commonly known as Sweta Arka (English), Madar (in Hindi), Aakand (in Bengali), Alark (in Sanskrit), Erukku (in Tamil) and Rui or Aak (in Marathi) etc. is a highly medicinal plant species of the Indian Himalayan region. The plant is native of India and other Asian countries like Egypt, China and Malaysia while its distribution is also recorded from Afganistan, Thailand, Nepal, Saudi Arabia, Pakistan, Thailand, Virgin Islands etc[8]. The plant is distributed upto 900 m in India, whereas its distribution is also observed from the foothills of Shivalik hills covering areas of six districts (Sirmour, Hamirpur, Chamba, Solan, Bilaspur and Kangra) of Himachal Pradesh[9-10]. *C. gigantea* is an important ethnomedicinal plant in the state. Plant leaves have anti-inflammatory, antimicrobial and antioxidant activities, whereas grounded powder of flowers is useful in the treatment of inflammation, asthma and tumors etc[11-12].Additionally, plant roots have antipyretic and antihepatotoxic activity against cold and cough[13,11]. The laticiferous fluid of *Calotropis* plant possesses proteolytic activity due to the presence of cysteine proteinase and aspartic proteinase enzyme, which makes them resistant against phytopathogens and insects[14].

Researchers have taken interest only on the pharmacology and biochemistry of *C.gigantea* but the genetic diversity of the wild population has rarely been explored from Himachal Pradesh, India. Therefore, current study was focused on study of intraspecific morphological and genetic variations within the population of *C. gigantea* from the Shivalik hills using morphological and molecular markers.

MATERIAL AND METHODS

SITE SELECTION AND PLANT COLLECTION

The altitude of the Shivalik hills of Himachal Pradesh vary from 350 meters to 1500 meters above the mean sea level. Therefore, five districts of Himachal Pradesh (Sirmour, Una, Solan, Kangra and Hamirpur) within the range of Shivalik hills (upto 900 meters) were selected for sample collection (Fig.1). The plant parts (branches, leaves, flowers and fruits) of three *C. gigantea* species were collected from five districts using purposive sampling method due to very less availability of the plant in the state. Identification of plants was done in the field during flowering season (April).

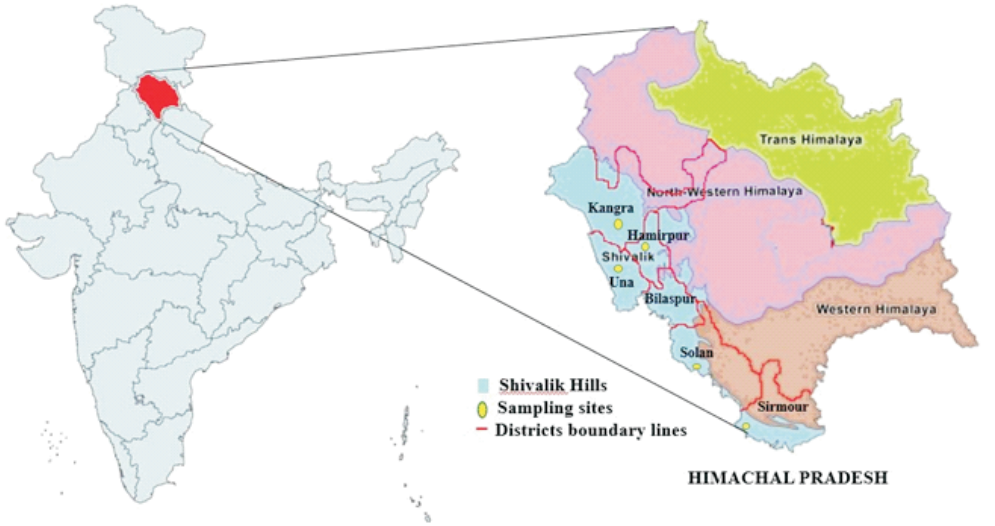

Fig. 1: Map of Himachal Pradesh, India showing the Shivalik hills and sample area

MORPHOLOGICAL ANALYSIS

The morphological features were studied in the month of April. Plants of five districts having voucher specimen numbers SUBMS/BOT-5225-A, 5225-B, 5225-C, 5225-D and 5225-E were deposited in the herbarium of Shoolini University, Solan. Different qualitative characters of the plant were studied which includes habit of plant, external shape of stem, leaf characteristics (leaf type, shape, margin, apices, surface, venation, phyllotaxy), flower characteristics (bract and bracteoles, attachment of flower, symmetry, presence of reproductive organs, number of floral parts, arrangement of floral organs, color, calyx, corolla, androecium, gynoecium and placentation), type of fruit and seeds etc.

RAPD ANALYSIS

GENOMIC DNA ISOLATION AND QUANTIFICATION

Genomic DNA was isolated from young flower petals of the selected *C. gigantea* plants by following CTAB method with some modifications[15]. The quality and quantity of isolated DNA was evaluated by agarose gel electrophoresis and UV-visible spectrophotometer (Systronics, India), respectivey. The extracted DNA was further stored at -80ºC for further PCR amplification.

PRIMER SELECTION

Five random primers of Operon Technology, USA were selected from the previous study done on this plant[16]. Only those primer were selected which have shown more than 60% polymorphism. The selected primers with their nucleotide sequences are listed in Table 1.

PCR AMPLIFICATIONS

The amplification reactions were performed in total volume of 25µl containing 1X PCR buffer, 25mM $MgCl_2$, 200µM each of dATP, dCTP, dGTP and dTTP (Medox Biotech, India), 15ng of random primers and 0.5U of *Taq* DNA polymerase (MP Biomedical, India). For each amplification reaction, 20ng of template DNA was used in Thermal Cycler (Labnet International, USA) programmed for four parameters. Initial denaturation was performed at 94ºC for five minutes. Denaturation step was performed at 94ºC for one minute followed by primer annealing and extension at 34ºC for two minutes and at 72ºC for two minutes, respectively. These steps were performed for 42 cycles. An additional extension at 72ºC for seven minutes was performed to promote complete extension. The PCR products after amplification were separated on 1.5% Agarose gels using 1X TAE buffer. After electrophoresis, gel was viewed under a UV transilluminator (Genaxy, India) and photographed by a Gel Doccument system (Alpha Innotech, USA). Amplification with each primer was repeated at least twice in order to ensure reproducibility.

DATA ANALYSIS

The RAPD data banding patterns were scored on the basis of presence "1" and absence "0" of each amplified band. To perform cluster analysis of RAPD, NTSYS-pc, version 2.02 software was used. Similarity between accessions was estimated using the JACCARD's coefficient. Unweighted pair group method with arithmetic averages (UPGMA) was used to calculate similarity estimates and resulting clusters were expressed as dendrogram for *C. gigantea* plants.

Table 1: List of primers with nucleotide sequences

Primer	Sequence
OPM-12	5'-GGGACGTTGG-3'
OPM-13	5'-GGTGGTCAAG-3'
OPM-14	5'-AGGGTCGTTC -3'
OPH-18	5'-AGGGTCGTTC-3'
OPAK-14	5'-CTGTCATGCC-3'

RESULTS

MORPHOLOGICAL ANALYSIS

The morphological variations of ten genotypes of *C. gigantea* from five different sites based upon qualitative characters are presented in Table 2 and Fig.2. All selected trees of *C. gigantea* from five sites were found to possess habit of shrub or a small tree. The shape of all trees were found to be cylindrical and no variations were observed from all sites**.** All trees possessed erect stem with pale grey bark. Leaves were observed opposite, decussate, simple with entire margins, sessile, stipule absent, blade ovate and reticulate unicostate venation. Flowers were bracteate, complete, bisexual, actinomorphic, pedicellate and white in colour. Sepal were five, free, lobed, shortly untied at the base and aestivation was quincuncial. Petals were also five, fused, five lobed with twisted aestivation. Stamen were five, gynandrous with dithecous anthers. Ovary was bicarpellary, apocarpous and style was united at their apex. Stigma was peltate with five lateral stigmatic surface. Anther adenate to stigma forming gynostegium. There was one variation observed w.r.t. calyx characters in district Hamirpur (Fig. 3). It was observed that *C. gigantea*, collected from Hamirpur district had one larger calyx than the others, whereas that character was absent in other plants collected from other districts. Fruits were observed to be etario of follicle. Thus there was no variation was observed related to this characters. Seeds were numerous, broadly ovate. Flat tuft of silk hair were present on the seeds of all genotypes.

RAPD ANALYSIS

In the present study, a set of five random decamer primers were used to observe intraspecific genetic variations between the

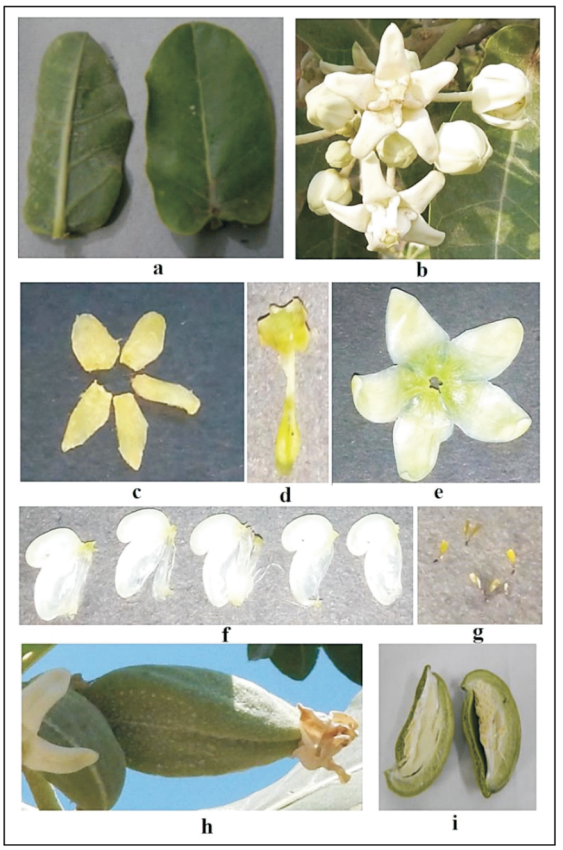

Fig. 2: *C. gigantea* (a) Leaves; (b) Flowers; (c) Calyx; (d) Stigma; (e) Corolla; (f) Stigmatic surfaces; (g) Pollinia; (h) Fruit; (g) Seeds in fruit

Table 2: Qualitative characters of *C. gigantea* collected from different sites of Himachal Pradesh

C. gigantea	Characteristic features
Habit	Shrub or a small tree.
External shape **Stem** **Leaves**	Cylindrical shape. Stem erect with pale grey bark Opposite-decussate leaf arrangement, simple entire margins, sessile, stipule absent, blade ovate and reticulate unicostate venation.
Inflorescence	A dense, multiflowered, umbellate, peducled cymes, arising from the nodes and appearing axillary or terminal.
Flower	Bracteate, complete, bisexual, actinomorphic, pentamerous, hypogynous, pedicellate
Calyx	Sepal 5, polysepalous, five lobed, shortly untied at the base, quincuncial aestivation
Corolla	Petals five, gamopetalous, five lobed, twisted aestivation
Androecium	Stamen five, gynandrous, antherdithecous.
Gynoecium	Bicarpellary, apocarpous, styles were united at their apex, peltate stigma with five lateral stigmatic surface. Anther adenate to stigma forming gynostegium.
Fruit	An etario of follicle
Seed	Numerous broadly ovate, flat tuft of silk hair

Fig. 3: Parts of flower of *C. gigantea* collected from Hamirpur district (picture showing one larger sepal in calyx)

Table 3: Total number of bands generated by five random primers and their percentage polymorphism

Name of primer	Amplification size range	Total number of amplified bands	Total number of polymorphic bands	Total number of monomorphic bands	% Polymorphism
OPM-12	125-1000bp	6	5	1	83
OPM-13	110-900bp	6	5	1	83
OPM-14	200-1000bp	5	5	-	100
OPH-18	100-1400bp	8	6	2	75
OPAK-14	190-900bp	11	7	4	63
TOTAL		**36**	**28**	**8**	**77**

genotypes of *C. gigantea* collected from different district of Himachal Pradesh. All primers gave different RAPD patterns and bands were found to be ranged in molecular size between 100 to 1500 bp as shown in Table 3. Only the dominant scorable bands were calculated while the week bands were excluded. With these five random primers, a total of 36 bands were obtained, out of which 28 were polymorphic. Primers OPAK-14 and OPH-18 were observed to be most reproducible, since the numbers of bands produced by them were eleven and eight, respectively as shown in Fig. 4. While the minimum number of bands, i.e., five were produced by primer OPM-14. Additionally, primers OPM-12 and OPM-13 produced six bands each. Results showed 100% polymorphism with primer OPM-14 whereas 83% polymorphism was observed with primers OPM-12 and OPM-13 as shown in table 3. The overall percentage polymorphic loci in the present study were 77% among the five genotypes.

CLUSTER ANALYSIS

The primarily clustering of *C. gigantea* genotypes was performed on the basis of Jaccard's coefficient and shown in Fig. 5. Highest value of similarity coefficients of 0.67 was found between the genotypes of Sirmour and Solan districts and the genotypes of Una and Hamirpur districts, thus suggesting their similar nature. This was further followed by similarity values of 0.44 between the genotypes of district Kangra and districts Una or Hamirpur within the range of Shivalik hills, although they are known to share almost similar climatic conditions. A very low level of genetic similarity (0.32) was observed between the two groups i.e., genotypes of districts Solan and Sirmour are in one group and districts Una, Hamirpur and Kangra are in other group.

DISCUSSION

A characteristic feature of a living organism is the vast natural variability present for various characteristics in most populations. A wide range of variability present in a species, always provide a better chance of selecting desirable genotype. Human activities have dramatically accelerated the global rate of species extinction. Markers (morphological and molecular) offer a great potential in determining the genetic variation and have been efficiently used to give reliable and reproducible results for estimating the variations. Morphological markers are visually studied traits in terms of both qualitative and quantitative manner and are controlled by the mutual affect of environment and the genotype of the plant. Results of present study showed no variation in all the plants of *C. gigantea* w.r.t. quantitative characters (habit, external shape, stem, leaf, flowers, etc). A very little variation was observed in the plants of *C. gigantea* collected from Hamirpur district w.r.t. calyx shape (flowers had one larger calyx (sepal) than the others), shown in Fig.3, whereas, this character was missing in plants of other districts. Such types of variations in qualitative traits have been studied by different authors among different plant species, e.g., in *Pinus ponderso*, *Rosa camina, R. iberica, R. foetida and R. hemisphaerica,* etc.[17-18]. The morphological study on the basis of leaf shape, length of sepal, leaf venation in *Hoya parasitica* (member of Asclepiadaceae) revealed that anatomical and morphological variations occurred due to environmental differences of two situations (full or partial exposure to the sun and partial shade and moist habitat)[19]. Whereas study of morphological variations in four perennial species (*Dalbergia sissio, Delonix regia, Cassia fistula and Calotropis procera*) proved that such variations were

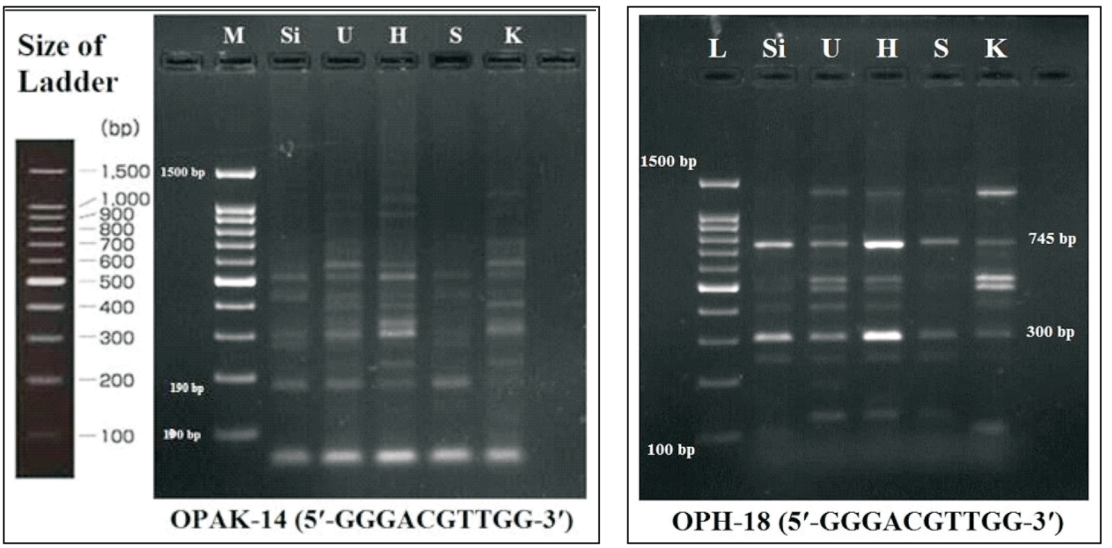

Fig. 4: RAPD profile generated by primers OPAK-14 and OPH-18 from genomic DNA of *C. gigantea* collected from Sirmaur (Si); Una (U); Hamirpur (H); Solan (S) and Kangra (K) district (M represents the ladder of marker having molecular size ranged between molecular 100-1500bp)

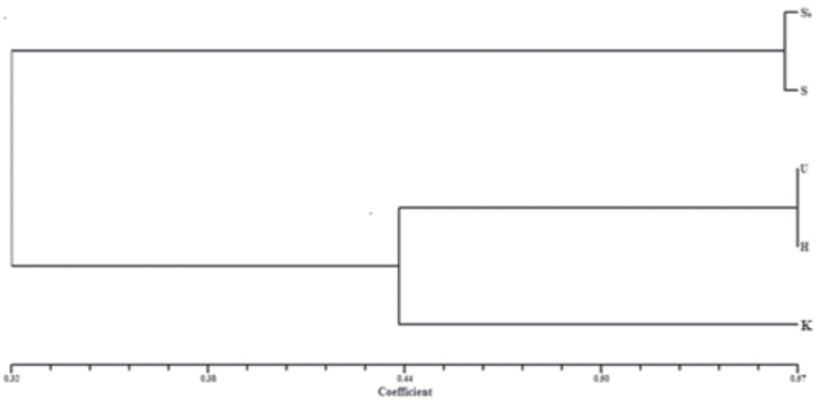

Fig. 5: Phylogenetic tree constructed by Jaccard's coefficient showing genetic relationship among populations of *Calotropis gigantea* in H, S, Si, U and K based on combined data of RAPD primers

due to varying demo edaphic factors[20].Similarly, intraspecific variation in *Calotropis species* regarding their leaf characters, plant height and corolla opening were found to be due to the reproductive isolation barrier[21]. In *Acer rubrum* the variations in leaf size and shape has been found to be influenced by ecological conditions including sunlight, water, and nutrient availability[22]. Hence literature proved that phenotypic variations in plants occur either due to different geographical conditions, altitudinal variation and demographic factors or it may be due to an epigenetic change. Therefore, from the above discussion present study revealed that the observed abnormal character could be the result of epigenetic change or any other gene regulatory mechanism.

RPAD markers offer a great potential in determining the genetic variation in plants. In this study, RAPD technique was found to be suitable in revealing intraspecific genetic variation between *C. gigantea* genotypes. Five random primers produced a total of 36 bands, out of which 28 were polymorphic. The highest proportion of polymorphism, i.e., 100% and 83% was observed with the primers OPM-14 and OPM-12, OPM-13, respectively. The overall percentage polymorphic loci in the present study were 77% among the five genotypes. This result was less similar to the previously reported results in ten different accessions of *C. gigantea* where observed percentage polymorphism was to be 16%[16]. Whereas, 24.22% and 48% polymorphism was observed in Asclepiadaceous species[23]. In *Pinus roxburghii* the genetic polymorphism has been found to be 89.58%[7]. RAPD technique has previously been used for intraspecific genetic variations in different plants species such as *Lycopresicon esculentum*, *Mullus surmuletus*, *Lippia* species, *Sclerotinia sclerotiorum*, *Capsicum* sp., *Oroxylum indicum*, *Urtica dioica*, *Mentha* sp., *Thunbergia laurifolia*, *Acer rubrum and Kaempferia galanga* etc[24-32,22,6].

The dendrogram using the JACCARD's coefficient revealed the presence of two distinct clusters, Sirmour and Solan into one cluster, whereas Una, Hamirpur and Kangra into another cluster (Fig.5).The former cluster was found to comprise genotypes of Solan and Sirmour into one group because of similar geographic area, climatic conditions and less distance between two sampling sites, etc. Additionally, Sirmour district is surrounded by Solan to the southwest, which may be the reason of intermixing of their

genotypes. The second cluster was further divided into two groups, therefore differentiate Kangra district genotypes from Una and Hamirpur genotypes. As Sirmour and Solan districts are separated from Hamirpur and Una by Bilaspur district therefore it creates a reproductive barrier between them and thus separates four districts genotypes into two different groups. The plants collected from Kangra district got clustered into separate group. It showed very less similarity coefficient with other genotypes which could be due to the separation of Kangra particularly 'Nurpur' village (sampling site) from Una and Hamirpur by the Satluj river and therefore, preventing the intermixing of genotypes. Additionally, locations also differ in terms of distance and also by little variation in soil conditions. Such differences resulted into natural selection of different ecotypes. Hence, a molecular marker based study using RAPD was done as initial step to check feasibility of variants. Among five primers used, four primers clearly differentiated the genotypes of *C. gigantea*, thus revealing a rich source of genetic diversity among selected variants.

CONCLUSION

The present study concludes that all the groups were closely related to each other and have been originated from the same parental line. High genetic polymorphism between the genotypes collected from different districts shows their adaptation to particular habitat. This study is a preliminary work to find out the genetic and morphological variations among the plants. Further study requires to intensify on higher molecular techniques with larger sampling size to find out the actual genetic variations between plants of *C. gigantea*.

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