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MOLECULAR CHARACTERIZATION OF PLANTS OF CUSCUTA *REFLEXA* ACCESSIONS USING THE RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MOLECULAR MARKER.

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Abstract: *Cuscuta reflexa* (Convolvulaceae) is a holoparasitic vine that attacks the aerial parts of many shrubs, trees, and is used immensely in the Indian system of medicine. In this study, the randomly amplified polymorphic DNA (RAPD) technique was employed for Molecular Characterization of six plants of Cuscuta *reflexa* Accessions Using the Random Amplified Polymorphic DNA (RAPD) Molecular Marker. the RAPD technique was used to analyse the genetic variations in *C.reflexa* populations collected from six different locations of North India. The RAPD primers were listed in Table 4.5.3 (A and B kits; 5 primers each) were procured from Operon Technologies (USA) and after initial screening, primers OPA02, OPA03, OPB07 and OPB17 were selected on the basis of profiles with each of the template DNA tested. All RAPD reactions were carried out with same cycling conditions and chemicals. Fragment sizes of the amplification products obtained using RAPD primers were estimated from the gel by comparision with standard molecular weight marker . The amplification profiles of total genomic DNA from six different populations with four random primers produced 95 consistent RAPD markers, ranging in size from 0.2 kb to 2.4 kb; out of which 10 were monomorphic. Pattern of RAPD profiles produced by the primer OPB07 are shown in the figure 4.5.1. From the amplified products of different primers used, two different groups of unique bands were observed. This observation clearly indicates that the populations of six accessions can be divided in to two clusters. These similarity coefficients were used to generate a tree from cluster analysis using UPGMA method (Figure 4.5.2). The cluster analysis indicates that the six different populations of *C.reflexa* are grouped in to two major clusters based on similiarity indicies. Among the six populations, three of each cluster showed the highest similarity index (92%). The present study provides evidences through RAPD data to show the occurence of genetic variations among different collections of *cuscuta reflexa* On the basis of the RAPD analysis, the chosen samples have been grouped under two clusters and labelled A and B. The present study showed maximum similarity indices and minor variations among the populations.

Keywords: *Cuscuta reflexa*, herbal medicine, molecular marker, RAPD, cluster, DNA

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INTRODUCTION

Cuscuta reflexa is a rootless, leafless perennial parasitic twining herb of Convolvulaceae family, commonly known as Akashvalli or Dodder. The plant is distributed worldwide and in India about 6 species are found. It has no chlorophyll and cannot make its own food by photosynthesis. It grows on thorny or other shrubs, sometimes completely covering the bushes and trees . It spread from one host to another, and on each victim, they twine and cling tightly with special branching organs called haustorium. Haustorium penetrate the host and connect to the host xylem as well as to the host phloem and absorb from it both water and elaborated food stuffs such as sugar and amino acid. It lives its entire life without attachment to the ground and grows with the help of seeds which are minute and produced in large quantities. Seeds have hard coating, and survive in the soil for 5-10 years or more. They sprout at or near the surface of the soil. The germination of seeds can occur without a host and for this it has to reach a green plant quickly. The herb grows towards the smell of nearby plants. If the host contains food which is beneficial for it, then it produces a haustorium that insert themselves into the vascular system of the host and then its original root will die. It can grow and attach itself to multiple plants. The *Cuscuta reflexa* is investigated for antitumor, antimicrobial, hepatoprotective, anticonvulsant, antioxidant, induced alopecia activities. Many chemical constituents have been isolated from *Cuscuta reflexa* such as cuscutin, amarbelin, beta-sterol, stigmasterol, myricetin, qurecetin, cuscutamine, luteolin, bergenin , The crude water extracts of *C. reflexa* exhibited anti-HIV activity which could be due to combinatory effects with compounds of different modes of action (Mahmood et al. 1997). The methanol extract of *C. reflexa* exhibited antibacterial and free radical scavenging activity . The petroleum ether extract of *C. reflexa* and its isolate is useful in treatment of androgen-induced alopecia by inhibiting the enzyme 5alpha-reductase. Another species of the same genus, known as *Cuscuta chinensis* Lam. is also a parasitic plant. The medicinal value of *C. chinensis* is entirely different from that of *C. reflexa*. The stem is used in the treatment of sore heads and inflamed eyes. The seed are aphrodisiac, demulcent, Diaphoretic and hepatic tonic.

MATERIAL AND METHODS

The genuine plants of *Cuscuta reflexa* (Convolvulaceae) was collected from distinct six different location from north india that is, (Muzaffarnagar, Haridwar, Deharadun, Kumau, Pantnagar, Nanital.) The Plants that was collected from different location of North India were aunthicated by Dr. Anju Pal . G.B.Pant University of Agriculture and Technology, Pantnagar, U.K

Isolation of genomic DNA

Fresh leaf samples (young leaves) were collected from the field of above study sites (1-2 month old) were used for the isolation of DNA. About 2 grams of leaves were cut in to small bits and

transferred to a prechilled mortar. The leaf tissue was frozen in liquid nitrogen and ground in to a fine powder. The powder was transferred to a centrifuge tube and added with 10 ml of preheated (65°C) extraction buffer containing 1.5% (w/v) hexadecyl or cetyl trimethyl ammonium bromide (CTAB) (Doyle and Doyle, 1987). 10 mM Tris HCL (pH 8.0), 1.4 M sodium chloride, 20 mM EDTA and 0.1% v/v 2-mercaptoethannol. The mixture was incubated in a waterbath for 30 min at 65°C with occasional mixing. Equal volume of chloroform: isoamyl alcohol (24:1) v/v was added, gently mixed for 15 min and centrifuged at 10,000 rpm for 20 min at room temperature (37°C). The clear aqueous phase was transferred to a new tube and an equal volume of isopropanol in cold ice was added and mixed gently by inversion until the DNA was precipitated out (10-20 sec). The precipitated DNA was hooked out using a sterile bent Pasteur pipette and air dried. The dried pellet was dissolved in 200-500 µl of TE (10 mM Tris-pH 8.0, 1mM EDTA pH 8.0). The contaminant RNA was eliminated from DNA by treating the DNA sample with RNase to a final concentration of 20 µg/ml. The sample was kept at room temperature for 15 minutes.

3.7.2. Purification of DNA

Genomic DNA, was purified by phenol: chloroform: isoamyl alcohol (25:24:1) extraction mixture. An equal volume of phenol: chloroform: isoamyl alcohol mixture was added to the DNA sample and mixed by repeated inversions. The mixture was centrifuged at 10,000 rpm for 10 minutes at room temperature and the aqueous phase was transferred carefully to a fresh eppendorf tube. To the aqueous phase, an equal volume of chloroform was added and centrifuged at 10,000 rpm for 2 minutes and the aqueous phase was transferred to another tube. To the aqueous phase 1/10th volume of 3 M sodium acetate (pH 8.0 for genomic and plastid DNA, pH 7.0 for DNA fragments) and two volumes of absolute ethanol was added and pelleted by centrifugation at 10,000 rpm for 5 min. After discarding the supernatant the resulting pellet was dissolved in nuclease free water and stored at -20°C.

3.7.3. Quantification of DNA

Genomic DNA and amplified products were quantified using UV spectrophotometer. The diluted DNA samples (1:250) were read at 260 nm and distilled water was taken as blank. The amount of DNA was calculated by using the following formula.

$$\text{Amount of DNA (µg/µl)} = \frac{\text{OD(260nm)} \times 50 \times \text{dilution factor}}{1000}$$

3.7.4. PCR amplification

About 50 ng of DNA samples were taken in PCR tubes and mixed with 200µM of each dNTPs, 0.5M RAPD primer (Operon Technologies, Almanda, California). 25 mM MgCl2, 1 unit of *Taq* polymerase and reaction buffer (Genei, Bangalore, India). Finally the total reaction volume was made up to 25µl by nuclease free water. The reaction tubes were placed in MJ thermal cycler using the following cycling conditions.

Initial denaturation - 95°C for 3 min

Denaturation - 94°C for 1 min

Primer annealing - 37°C for 1 min

Extension - 72°C for 1 min and 20 sec. for 40 cycles

Final extension - 72°C for 15 min and then hold on at 4°C

3.7.5. Electrophoresis of samples

After the completion of PCR amplification, the samples were added with 2µl of loading dye containing TBE buffer, glycerol and bromophenol blue. Agarose gel (1.5%) was casted with 1X TBE buffer and the samples were loaded in the wells. Electrophoresis was carried out at 60 volts for 4 hours. After electrophoresis the gels were documented in Gel documentation system (Vilber Lourmet, France). The amplification product‟s size was calculated by using the software photocapt MW.

3.7.6. Primer screening

Primers were selected on the basis of the number and intensity of polymorphic amplified bands. Ten random primers from Operon Technologies (Almanda, California) were initially screened and using in different populations of *C.reflexa* to determine the suitability of each primer for the study. Primers were selected for further analysis based on their ability to detect distinct, clearly resolved and polymorphic amplified products within the populations of *C.reflexa*. To ensure reproducibility, the primers generating no/weak/complex patterns were discarded. The primers were tested on the 6 plant populations of *C.reflexa*. From this, four primers were selected for further studies on the basis of good DNA amplification, with at least three sharp electrophoretic bands.

3.7.7. Agarose gel electrophoresis

Required amount of agarose was weighed out (0.75% for genomic DNA and 1.5% for amplified products) and melted in IX TBE buffer (90 mM Tris-borate and 2mM EDTA-pH 8.0)) or IX TAE

buffer (40 mM tris-acetate, 1mM EDTA-pH 8.0). After melting, the volume of the gel mixing was made up to the final volume by the addition of water. After cooling to 50°C, ethidium bromide was added to a final concentration of 0.5 mg/ml. The mixture was poured immediately on a preset template with appropriate comb. After solidification, the comb and the sealing tapes were removed and the gel was mounted in an electrophoresis tank. To the DNA sample, required volume of sample buffer (6X sample buffer : 40% sucrose, 0.25% bromophenol blue) was added and the samples were loaded onto the gel. Electrophoresis was performed at 60 volts for 4 hours.

RAPD data analysis

PCR products from individual plants were scored as either present or absent. Only clearly amplified fragments were analysed. Scores of 1 (present) or 0 (absent) were used to form a matrix. The genetic distance was calculated as the precentage of total number of bands scored that were clearly different between each pair of accessions. Each amplification fragmets was named by the source of the primer (Operon, Advanced Biotechnologies) the kit letter or number, the primer number and its approximate size in base pairs. Similarity indices were estimated using the Dice coefficient of similarity (Nei and Li, 1979). Cluster analysis was carried out on similarity estimates using UPGMA (unweighed pair-group method to produce a dendrogram using arthimetic average) in the NTSYSpc-version 1.80 software program (Rohlf, 1993).

RESULT & DISCUSSION

Molecular characterization of six accessions of *Cuscuta reflexa* using RAPD analysis

All the collected six populations from different parts of Uttarpradesh and Uttrakhand,North India were maintained under uniform growth conditions and used for RAPD analysis. In the present study, the RAPD technique was used to analyse the genetic variations in *C.reflexa* populations collected from six different locations of North India. The RAPD primers were listed in Table 4.5.3 (A and B kits; 5 primers each) were procured from Operon Technologies (USA) and after initial screening, primers OPA02, OPA03, OPB07 and OPB17 were selected on the basis of profiles with each of the template DNA tested. All RAPD reactions were carried out with same cycling conditions and chemicals. Fragment sizes of the amplification products obtained using RAPD primers were estimated from the gel by comparision with standard molecular weight marker (DNA ladder from Genei, Bangalore).

Among the different primers screened, four primers showed good amplification of polymeric bands (Table 4.5.4) and three did not give any amplification products. Three of the primers showed amplifications but the intensity of the fragments was very low. The amplification

profiles of total genomic DNA from six different populations with four random primers produced 95 consistent RAPD markers, ranging in size from 0.2 kb to 2.4 kb; out of which 10 were monomorphic. Pattern of RAPD profiles produced by the primer OPB07 are shown in the figure 4.5.1. From the amplified products of different primers used, two different groups of unique bands were observed. This observation clearly indicates that the populations of six accessions can be divided in to two clusters. The cluster A includes populations of Muzaffarnagar, Haridwar, and Deharadun while cluster B comprises the populations of Kamau, Pantnagar and Nainital.

The similarity indices as given in table 4.5.5, which clearly shows *Cuscuta reflexa* accessions from the North India show less varitions. Even though they may be belonging to geographically distinct locations, they are very close nearly to 92% in terms of similarity index. The similarity coefficient values range from 0.88 to 0.92 of six different populations of *Cuscuta reflexa*. These similarity coefficients were used to generate a tree from cluster analysis using UPGMA method (Figure 4.5.2). The cluster analysis indicates that the six different populations of *C.reflexa* are grouped in to two major clusters based on similiarity indicies. One major cluster had three members in the population *i.e.,* Muzaffarnagar, Haridwar and Deharadun. Another major cluster includes three populations *viz*., Kumau, Pantnagar and Nainital. Each and every population could be identified by using four random 10-mer primers. Among the six populations, three of each cluster showed the highest similarity index (92%). The present study provides evidences through RAPD data to show the occurence of genetic variations among different collections of *cuscuta reflexa* On the basis of the RAPD analysis, the chosen samples have been grouped under two clusters and labelled A and B. The present study showed maximum similarity indices and minor variations among the populations.

Fig 4.5.1 - RAPD fingerprints of *Cuscuta reflexa*, obtained from PCR amplification using Primer OPB 7

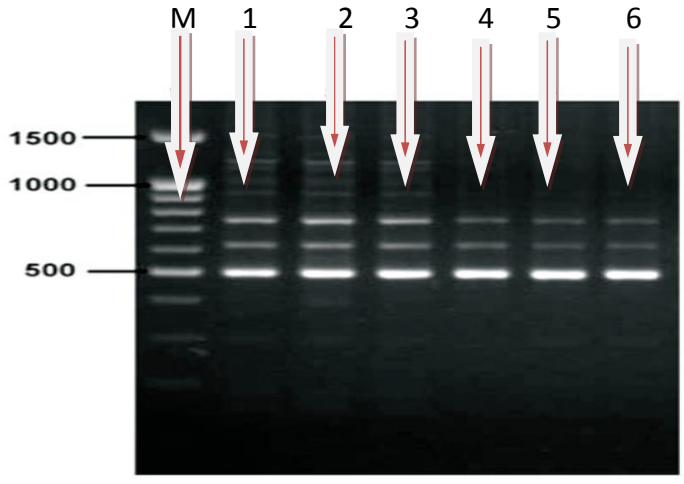

Fig. 4.5.2. UPGMA dendrogram showing the genetic relationships within six different population (location) of *Cuscuta reflexa*

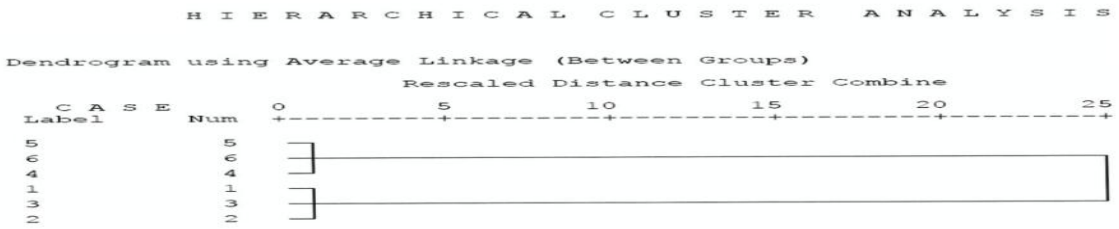

Table 4.5.3. Sequences of the random nucleotide primers.

S.No	Primer	Sequences
1	OPA01	CAGGCCCTTC
2	OPA02	TGCCGAGCTG
3	OPA03	AGTCAGCCAC
4	OPA07	GAAACGGGTG
5	OPA12	TCGGCGATAG
6	OPB04	GGACTGGAGT
7	OPB07	GAAACGGGTG
8	OPB12	CCTTGACGCA
9	OPB15	GGAGGGTGTT
10	OPB17	GACCGCTTGT

Table 4.5.4. . Total number of amplified fragments and number of polymorphic bands generated by PCR using selected random decamer in six populations (locations) of *Cuscuta reflexa.*

S.No	Name of the primer	Sequence of the primers	Total number of amplification products	Number of polymeric products	Size range (kb)
1	OPA02	TGCCGAGCTG	5	2	0.8-1.6
2	OPA03	AGTCAGCCAC	10	6	0.2-1.8
3	OPB07	GAAACGGGTG	8	8	0.6-2.1
4	OPB17	GACCGCTTGT	10	6	0.6-2.4

Table 4.5.5. Similarity index of *Cuscuta reflexa* accessions using RAPD

	MUZ	HAR	DEH	KUM	PAN	NAI
Muzaffarnagar	1.0					
Haridwar	0.91	1.0				
Deharadun	0.88	0.86	1.0			
Kumau	0.76	0.68	0.80	1.00		
Pantnagar	0.72	0.76	0.88	1.00		
Nainital	0.76	0.67	0.74	0.92	0.92	1.00

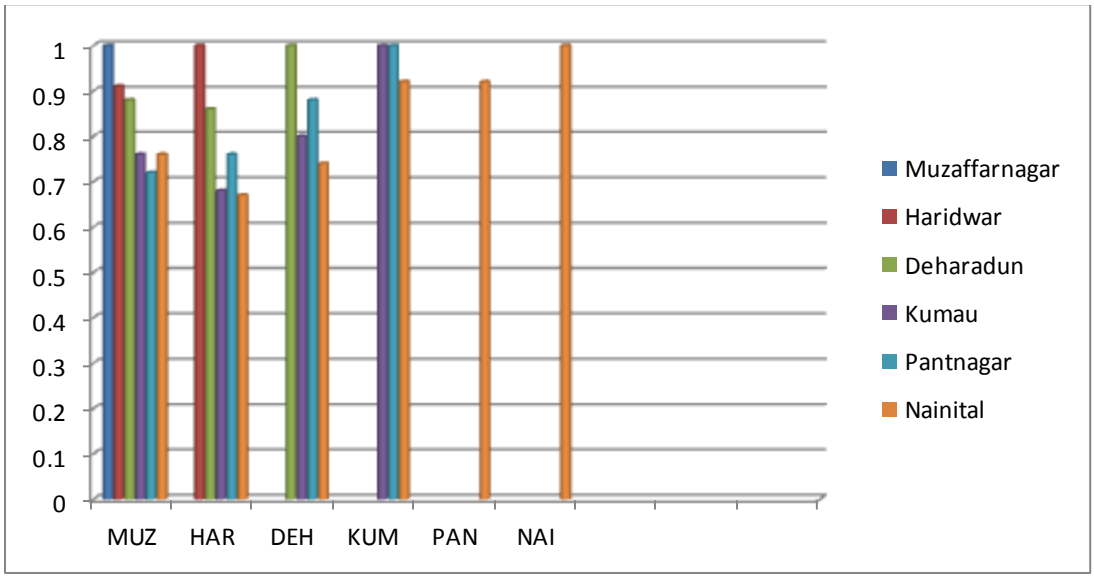

Fig 4.5.6 - Grappical representation of Similarity index of *Cuscuta reflexa accessions* using RAPD

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REFFERENCES

1. Khan S, Mirja KJ, Md. Tayyab, Abdin MZ (2009) RAPD profile for authentication of medicinal plant *Glycirrhiza glabra* L. Medicinal and Aromatic Plant Science and Biotechnology 3, 48-51.
2. Khan S, Mirza KJ, Anwar F, Abdin MZ (2010) Development of RAPD markers for authenticationof *Piper nigrum*. Environment & International Journal of Science and Technology 5, 53-62.
3. Khan S, Qureshi MI, Kamaluddin, Tanweer A, Abdin MZ (2007) Protocol for isolation of genomic DNA from dry and fresh roots of medicinal plants suitable for RAPD and restriction digestion. African Journal of Biotechnology 6, 3, 175-178.
4. Acharya L, Mukherjee AK, Panda PC, Das P (2005) Molecular characterization of five medicinally important species of *Typhonium* (Tracheae) through random amplified polymorphic DNA (RAPD). Zeitschrift für Naturforschung C, 7-8, 600-604.
5. Ma HX, You ZL, Wang XY (2008) Effect of total flavones from *Cuscuta chinensis* on expression of Fas/FasL, PCNA and HB-EGF in SD rats model with bromocriptine-induced abortion. Zhong Yao Cai 31, 11, 1706-1709.
6. Mahmood N, Piacente S, Burke A, Khan A, Pizza C (1997) Constituents of *Cuscuta reflexa* are anti-HIV agents. Antiviral Chemistry and Chemotherapy 8, 70-74.
7. Shinde M, Dhalwal K, Mahadik KR, Joshi KS, Patwardhan BK (2007) RAPD analysis for determination of components in herbal medicine. Evidence based Complimentary and Alternative Medicine 4, 21-23.
8. Zhen GH, Jiang B, Bao YM, Li DX, An LJ (2006) The protect effect of flavonoids from *Cuscuta chinensis* in PC12 cells from damage induced by H2O2. Zhong Yao Cai 29, 10, 1051-1005.
9. Pal DK, Mandal M, Senthilkumar GP, Padhiari A (2006) Antibacterial activity of *Cuscuta reflexa* stem and *Corchorus olitorius* seed. Fitoterapia 77, 589-591.
10. Pandit S, Chauhan NS, Dixit VK (2008) Effect of *Cuscuta reflexa* Roxb on androgen-induced alopecia. Journal of Cosmetic Dermatology 7, 3, 199-204.
11. McClelland M, Mathieu-Daude F, Welsh J (1995) RNA fingerprinting and differential display using arbitrarily primed PCR. Trends in Genetics 11, 242-246.
12. Meherotra S, Rawat AKS (2000) Standardization and quality evaluation of herbal drugs In: Chauhan DK (ed.), Recent Trends in Botanical Research, Dikshit Press, Allahbad, 313-332.
13. Micheli MR, Bova R, Pascale E, Ambrosio ED (1994) Reproducible DNA fingerprint with the random amplified polymorphic DNA (RAPD) method. Nucleic Acids Research 22, 1921-1922.
14. Na H, Um J, Kim S, Koh K, Hwang W, Lee K, Kim C, Kim H (2004) Molecular discrimination of medicinal *Astragali radix* by RAPD analysis. Immunopharmacology and Immunotoxicology 26, 265-272.