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Genetic Diversity Analysis and Molecular Representation of *Cuscuta reflexa* Population Using RAPD (Random Amplified Polymorphic DNA) Molecular Marker

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ABSTRACT

Cuscuta reflexa Roxb. stem samples were collected from six ecologically distinct regions of North India and analyzed for genetic diversity using RAPD (Random Amplified Polymorphic DNA) markers, alongside phytochemical profiling of ethanolic extracts. Of the 10 decamer primers screened, four (OPA02, OPA03, OPB07 and OPB17) produced distinct and reproducible amplification patterns, generating a total of 95 bands, of which 85 were polymorphic and 10 monomorphic. The band size ranged from 0.2 to 2.4 kb. Cluster analysis based on UPGMA grouped the six populations into two major genetic clusters, revealing a high similarity index (up to 92%) among geographically proximal populations such as Muzaffarnagar, Haridwar and Dehradun, and comparatively lower similarity with the Kumaun, Pantnagar and Nainital populations. Phytochemical screening of the ethanolic extract showed the presence of major secondary metabolites including flavonoids, tannins, phenolics, glycosides, saponins, triterpenoids, amino acids and steroids, with ethanol yielding the highest extractive value (12.51% w/w). These results not only demonstrate significant intraspecific genetic variability in *Cuscuta reflexa* but also validate the rich phytochemical composition of its ethanolic extract, supporting its pharmacological potential.

Key words: *Cuscuta reflexa*, RAPD analysis, genetic diversity, population structure, molecular markers, cluster analysis

INTRODUCTION

The genus *Cuscuta*, which is comprised of plants that are parasitic, has garnered the attention of the scientific community due to the extensive number of medical applications that it possesses. This particular species is leafless, rootless, perennial parasitic twining herb belonging to the family Convolvulaceae (Akinyede *et al*., 2020). It is also popularly referred to as Akashvalli or Dodder *Cuscuta reflexa*. However, it does not have any roots or leaves. India is home to six different species of this species; however it has been documented in every region of the planet. According to Rathore *et al*.'s research from 2020, *C. reflexa* is a parasite since it is unable to photosynthesize and, as a result, does not possess chlorophyll. It flourishes on thorns or other shrubs, and it can occasionally fully surround the bushes and trees. By connecting itself between the two plant hosts through the use of specialized branched structures known as haustoria, *C. reflexa* is able to propagate that organism onto each new plant (Dar *et al*., 2020). *C. reflexa* attaches itself to the host as an epiphyte and penetrates the host's tissues by penetrating both the xylem and the phloem. Once inside, it extracts water, nutrients and complex foods such as carbohydrates and amino acids from the host. In a manner that is somewhat counterintuitive, the life cycle of *C. reflexa* is noteworthy in that it is grown while connected to one or more plants throughout its whole life cycle, and it will never touch the ground. It is possible for *C. reflexa* to produce a large number of seeds, each of which is very minute. These seeds are protected by a tough coating, which allows them to remain in the soil for at least 5 to 10 years. Furthermore, the plant is known to develop towards the smell of neighbouring plants in order to reach a green plant in order to initiate a parasitic link. These seeds are capable of germinating even in the absence of a host. It has been well established and

reviewed in previous research that *C. reflexa* possesses a wide range of activities, including antitumor, antimicrobial, hepatoprotective, anticonvulsant, antioxidant activity and alopecia induction. The medicinal value of *C. reflexa* is multi-directional and possesses a broad range of activities (Harfiani *et al*., 2022). In addition to these, it has been reported that *C. reflexa* has a number of bioactive substances, including cuscutin, amarbelin, beta-sterol, stigmasterol, myricetin, quercetin, cuscutamine, luteolin, bergenin and essential oils. These chemicals have been extracted from the plant. Despite the fact that these compounds have been isolated, it is unexpected to learn that crude water extracts of *C. reflexa* have also demonstrated effectiveness against HIV. This is likely due to the influence of the combinatory action of the plant's various components working together in synergy (Ramya and Devika, 2022). This particular species is currently the focus of a significant amount of scientific attention, with the objective of identifying its unusual ecological adaptability, as well as its widespread medicinal value that encompasses a wide range of pharmacological actions (Qu and Zhang, 2020). Using Random Amplified Polymorphic DNA (RAPD) markers, the current study compared the genetic diversity and molecular characterization of different populations of *C. reflexa* that were collected from different locations in North India. The objective of the study was to categorize the different *C. reflexa* populations into distinct clusters.

MATERIALS AND METHODS

The plant *Cuscuta reflexa* was collected from six geographically distinct locations in North India: Muzaffarnagar, Haridwar, Dehradun, Kumaun, Pantnagar and Nainital. Identification of the species was conducted using available floristic literature, relevant research articles and by comparing voucher specimens with authenticated herbarium records (Shree *et al*., 2019; Panda and Barik, 2021; Mahendru *et al*., 2022). Fresh tissues were collected from all locations and immediately stored in cooling boxes containing ice packs to preserve their integrity during transportation. In the laboratory, the samples were thoroughly cleaned, labelled, wrapped in aluminum foil and kept frozen until further use. For genetic analysis, an initial screening of 10 random primers designed by Operon Technologies (Almadena, California) was carried out to identify effective primers suitable for DNA amplification (Table 1).

Table 1. Random primer used for amplification of DNA

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

Six populations of *C. reflexa* were tested, and based on amplification profiles, four primers showing good results were selected for further investigation (Umar *et al*., 2023). Following sample collection, the parasitic *C. reflexa* stems were carefully separated from the host plants and thoroughly washed to remove dirt and other contaminants. The cleaned material was shade-dried for 6 - 8 days until complete dehydration and then powdered using a mechanical grinder.

For extraction, the maceration method was employed. Eighty grams of the powdered plant material was soaked in 400 ml of ethanol in a conical flask. This mixture was left undisturbed for 48 h with occasional stirring. After the maceration period, the mixture was filtered initially through a four-fold muslin cloth and subsequently through Whatman No. 1 filter paper. The filtrate was then evaporated to dryness at 40^0C. The yield and physical characteristics, such as colour, of the extract were recorded, and the dried extract was stored at 4^0C in a refrigerator for further analysis (Raghavendra *et al*., 2021).

For the purpose of this investigation, approximately two grams of the whole plant samples were meticulously cut into small pieces. The samples were subsequently weighed in a mortar that had been pre-chilled using liquid nitrogen and then ground into a fine powder. Subsequently, the powder was placed into a centrifuge tube, followed by the addition of 10 ml of a CTAB extraction buffer, which was preheated to 65°C. The buffer comprised the following components: A weight-

to-volume ratio of 1.5% CTAB, 10 millimolar Tris-HCl at a pH of 8.0, 1.4 millimolar NaCl, 20 millimolar EDTA and 0.1% volume-to-volume 2-mercaptoethanol. Subsequently, the mixture was subjected to a water bath maintained at 65°C for 30 min, during which interactions occurred intermittently. Following a careful mixing for 15 min, an equal volume of chloroform isoamyl alcohol (24:1) was introduced, and the resulting mixture was subsequently centrifuged at 10,000 revolutions per min for 20 min at room temperature. The clear aqueous phase was meticulously transferred to a different tube, followed by the addition of equal volumes of isopropanol that had been cooled to -4°C. The liquid was inverted manually for duration of 10-20 sec, allowing the DNA to precipitate during this period. Following this, the precipitated DNA was extracted utilizing a sterile, bent Pasteur pipette. Subsequently, it was permitted to air-dry before being re-suspended in a TE buffer containing 10 mM Tris, 1 mM EDTA and adjusted to a pH of 8.0. Following the treatment of DNA with RNase at a final concentration of 20 µg/ml an incubation period of 15 min at room temperature was observed. This procedure was implemented to remove any remaining RNA residues. The combined mixture was incubated in the water bath for 30 min at a temperature of 65°C, with minimal mixing applied throughout the process. A precise volume of chloroform isoamyl alcohol (24:1) was thoroughly mixed into the faecal suspension medium for duration of 15 min. Subsequently, the mixture underwent centrifugation for 20 min at a speed of 10,000 revolutions per min, maintained at room temperature (37°C).

The PCR reactions were conducted in a tube with a volume of 25 microliters. The PCR tubes contained approximately 50 ng of DNA samples, mixed with 200 ìM of each dNTP, 0.5 M RAPD primer (Operon Technologies, Almanda, California), 25 mM $MgCl_2$, 1 unit of Taq polymerase (and reaction buffer from Genei, Bangalore, India), along with a reaction buffer. The total reaction volume was reduced to 25 microliters using nuclease-free water. The PCR reaction mixture for each sample comprised 15 microliters of sterile distilled water, 1.5 microliters of $MgCl_2$ (50 mM), 2.5 microliters of 10X buffer, 0.5 microliters of Taq DNA polymerase (5 units per microliter), 2 microliters of primer (10 pmol / microliter), and 1 microliter of template DNA (10 to 30 nanograms per microliter). Denaturation occurred at 95°C for 3 min, succeeded by 40 cycles comprising denaturation at 94°C for one min, annealing at 36°C for one min, and elongation at 72°C for one min. The final extension occurred at 72°C for 7 min, with the temperature held at 4°C while awaiting the samples.

A PCR amplicon obtained from a DNA sample was utilized in this experiment. The amplicon was placed in a 1% agarose gel along with 0.5 ìl of loading dye. In order to separate the fragments, the gel was subjected to electrophoresis at a voltage of 80 volts for a period of minimum 4 h. In order to visualize the gel and take photographs of it, an ultraviolet (UV) trans-illuminator was utilized after the lanes and bands had finished separating. In every instance, there was a sample that did not contain any template DNA that was run as a negative control for contamination. Additionally, there was a DNA ladder that was used as a reference for the sizes of DNA fragments. It was determined how many polymorphic bands and monomorphic bands were present for each primer that was utilized. The PCR reactions were done in a PCR tube that contained 25 microliters. About 50 ng of DNA samples were loaded into these PCR tubes; thereafter, they were combined with 200 ìM of each dNTP, 0.5 M RAPD primer (Operon Technologies, Almanda, California), 25 mM MgCl2, one Taq polymerase unit, and reaction buffer. Using nuclease-free water, the total volume of the reaction was lowered down to 25 microliters. The total PCR reaction mixture for each of the samples consisted of 15 microliters of sterile distilled water, 1.5 microliters of MgCl2 (50 mM), 2.5 microliters of 10X buffer, 0.5 microliters (5 units per microliter), 2 microliters (10 pmol / microliter) of primer, Taq DNA polymerase and 1 microliter (10 to 30 nanograms per microliter) of template DNA. The process of denaturation was executed at 95°C for duration of 3 min. This was succeeded by 40 cycles comprising denaturation at 94°C for one min, annealing at 36°C for one min, and elongation at 72°C for one min. The concluding extension was executed at a temperature of 72°C for 7 min, while the samples were held at a constant 4°C during the waiting period.

Each plant's PCR products were evaluated to see if they were present or not. The only bands that were scored were those that were noticeably amplified. A matrix was developed, with a score of 1 corresponding to a band that was present and a score of 0 indicating that the band was not there. The calculation of genetic distance between individual accessions was derived from the percentage of the total number of scored bands that exhibited visible differences between each pair of accessions. Each amplification fragment was identified by referencing the origin of the primer (either Advanced Biotechnologies or Operant), the designation of the kit, the specific primer number and an estimated size in base pairs (bp). The Dice coefficient of similarity was employed in the calculation of similarity indices. Cluster analysis was conducted on the similarity estimations utilizing the NTSYSpc-version 1.80 software program by the UPGMA method, an acronym for the unweighted pair-group method with arithmetic average.

In the present study, stem samples of *C. reflexa* Roxb. were subjected to successive solvent extraction using petroleum ether, chloroform, ethyl acetate, ethanol and water. Preliminary phytochemical screening of the ethanolic extract was conducted to identify the presence of secondary metabolites such as alkaloids, flavonoids, saponins, terpenoids, glycosides, tannins, phenols and steroids. The detection was carried out using standard qualitative protocols as described by Kalita *et al*. (2017). To test for alkaloids, 1% HCl was added to the extract, followed by the addition of Mayer's and Dragendorff's reagents; the formation of a precipitate indicated the presence of alkaloids. For flavonoids, 5 ml of dilute ammonia solution was added to the aqueous extract, followed by concentrated sulfuric acid (H_2SO_4); a yellow colour that faded upon standing confirmed flavonoids. To detect saponins, 20 ml of distilled water was added to the extract and shaken in a graduated cylinder for 15 min; a 1 cm foam layer confirmed saponin presence. The presence of terpenoids was tested by adding 2 ml of chloroform and 3 ml of concentrated H_2SO_4 to 2 ml of the extract; a reddish-brown layer at the interface indicated terpenoids. For glycosides, 3 mg of extract was mixed with anthrone reagent and a few drops of concentrated H_2SO_4, for a gentle heating in a water bath; a dark green colour confirmed their presence. Tannins were tested by adding 1% lead acetate to 5 ml of extract; the appearance of a yellow precipitate indicated tannins. To detect phenols, 3 ml of ethanol and a pinch of ferric chloride ($FeCl_3$) were added to 2 ml of the extract; a greenish-yellow colour confirmed phenolic compounds. Lastly, for steroids, 0.5 g of extract was treated with 2 ml of acetic anhydride and 2 ml of concentrated H_2SO_4; a colour change from violet to blue or green confirmed the presence of steroids.

RESULTS AND DISCUSSION

The RAPD analysis was performed on all six groups that were sampled from different regions of North Indian states of Uttarakhand and Uttar Pradesh, but were all placed in settings that were comparable to one another. Operon Technologies, located in the United States, was the source of the RAPD primers that is presented in Table 1. Following the preliminary screening, four primers were chosen for the amplification process. These primers were OPA02, OPA03, OPB07, and OPB17. The selection was based on the RAPD profiles of the entire template DNAs. The cycle conditions and reagents that were used in each and every RAPD reaction were identical. A molecular weight marker, which was a DNA ladder purchased from Genei in Bangalore, was utilized in order to determine the size of the fragments of amplified products obtained by RAPD. Out of the nine primers that were tested for RAPD analysis, four of them produced amplification products that were of a high quality for polymeric bands (Table 2), whereas

Table 2. The aggregate count of amplified fragments and the quantity of polymorphic bands produced through PCR utilizing a chosen random de-camer across six populations of *C. reflexa*

S. No.	Primer	Primer sequence	Total amplifications	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

the remaining three primers did not produce any amplification products. The amplification profiles of whole genomic DNA from the six populations were produced by the four random primers, which led to the production of a total of 95 RAPD markers. The diameters of the polymeric bands ranged from 0.2 to 2.4 kb. It was found that there were 10 monomorpic bands among the 95 RAPD markers.

Fig. 1 illustrates the RAPD profiles generated by the primer OPB07. The analysis of the amplified products from the various primers yielded two distinct band type groups. This indicated that the populations of the six accessions were categorized into two groups. Cluster A (1, 2, 3) included populations from Muzaffarnagar, Haridwar and Dehradun, whereas Cluster B (4, 5, 6) encompassed populations from Kumaun, Pantnagar and Nainital.

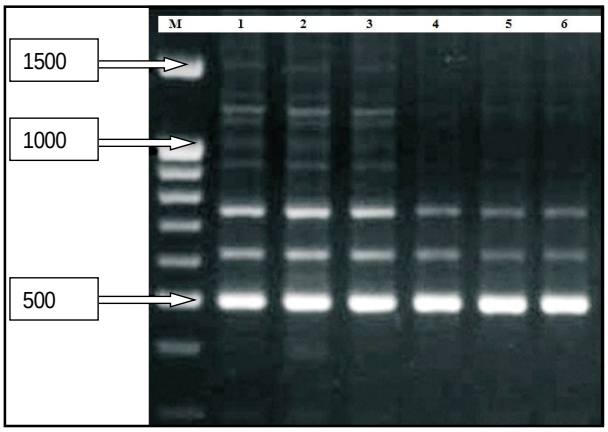

Fig. 1. RAPD fingerprints of *C. reflexa*, obtained from PCR amplification using primer OPB 7.

The similarity indices for all accessions of *C. reflexa* indicated that accessions from North India exhibited low variation (Table 3). Despite potentially originating from a distinct geographical area, their similarity was striking, with a relation of 92% according to the similarity index. The similarity coherence of six distinct populations of *C. reflexa*, as assessed through cluster analysis and the UPGMA method, ranged from 0.88 to 0.92.

The results of the cluster analysis indicated that the six populations of *C. reflexa* were suitable for grouping into two major clusters based on their similarity indices (Fig. 2). One major cluster consisted of the three populations of Muzaffarnagar, Haridwar and Dehradun. The other cluster had the three populations of Kumaun, Pantnagar and Nainital. No differentiation occurred using four random 10-mer primers with all populations having a (92%) similarity index to three from each cluster. It suggested that two main factors may account for the variation; the difficulty in homogenizing populations of *C. reflexa* when harvesting from a cuscutace of *Cuscuta* species that look alike, and the climatological environmental variation that complicated biological differences of these plants that developed in various geographic regions.

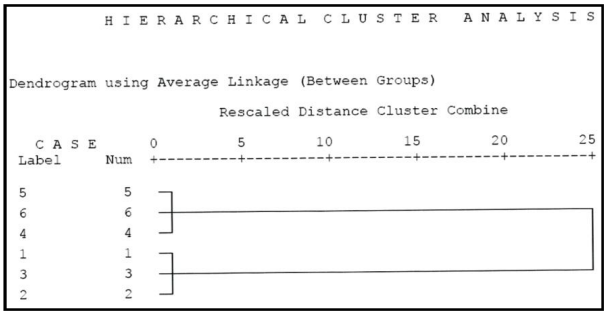

Fig. 2. UPGMA dendrogram showing the genetic relationships in six different populations (location) of *C. reflexa.*

The present study, utilizing RAPD data, supported the existence of genetic differences between the different collections of *C. reflexa.* The RAPD analysis of the selected samples clearly showed two groups or clusters A and B (Fig. 3). The previous study showed the highest similarity indices with the least variability in the populations.

The medicinal and pharmacological potential of plants is largely attributed to the presence of bioactive compounds known as secondary metabolites, including alkaloids, flavonoids,

Table 3. Similarity index of *C. reflexa* accessions using RAPD

	Muzaffarnagar	Haridwar	Dehradun	Kumaun	Pantnagar	Nainital
Muzaffarnagar	1.0					
Haridwar	0.91	1.0				
Dehradun	0.88	0.86	1.0			
Kumaun	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

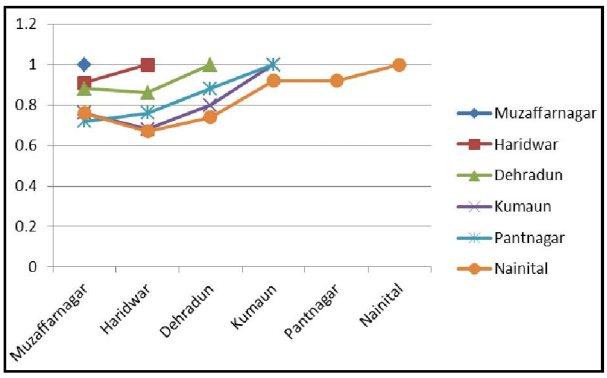

Fig. 3. Graphical representation of similarity index of *C. reflexa* accessions using RAPD.

tannins, saponins and terpenoids. These compounds collectively referred to as phytochemicals, play critical roles in plant defense mechanisms and also exhibit therapeutic effects in humans. In the present study, the stem samples of *C. reflexa* Roxb. were subjected to successive solvent extraction using various solvents such as petroleum ether, chloroform, ethyl acetate, ethanol and water. The percentage yield of extracts obtained from each solvent was recorded, revealing that ethanol and water extracts yielded the highest extractive values, while petroleum ether, chloroform and ethyl acetate yielded relatively low quantities (Table 4).

Table 4. Extractive values of the stem sample of *C. reflexa*

Extract type	Extractive value (% w/w)
Petroleum ether extract	0.80
Chloroform extract	0.85
Ethanol extract	12.51
Aqueous extract	6.88

Qualitative phytochemical screening of these successive extracts indicated the presence of several important secondary metabolites. The chemical tests revealed the occurrence of alkaloids, carbohydrates, saponins, flavonoids, tannins, phenolic compounds, steroids and triterpenoids in different solvent fractions (Table 5). The ethanolic extract, in particular, showed significant presence of most of these phytochemicals, supporting its use as an effective solvent for extracting bioactive constituents from *C. reflexa*. These findings suggest that *C. reflexa* is a rich source of various phytochemicals, many of which have known medicinal and therapeutic properties, and thereby validate its traditional use in herbal medicine.

CONCLUSION

The RAPD analysis of *Cuscuta reflexa* revealed clear genetic differentiation among the six North Indian populations, with clustering patterns strongly correlated with geographical origin. The high number of polymorphic bands and a similarity index ranging from 67 to 92% suggested adaptive divergence possibly driven by environmental and ecological factors. Simultaneously, the ethanolic extract of the stem displayed a high yield and a diverse range of bioactive phytoconstituents, reinforcing its medicinal significance. These findings collectively highlight *C. reflexa* as a genetically variable and phytochemically rich plant species, meriting further exploration for its therapeutic applications and conservation through genotype-based selection strategies.

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Table 5. Preliminary screening of phytochemicals present in the extracts of *C. reflexa* stem

Chemical compound	Petroleum ether	Chloroform	Ethanol	Water
Alkaloids	–	–	–	–
Glycoside (Anthraquinone)	–	+	+	+
Tannin	–	–	+	+
Flavonoids	–	–	+	+
Steroids	–	–	+	+
Triterpenoids	–	+	+	+
Saponin	–	–	–	+
Amino acids	–	–	+	+

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