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Full Length Research Paper

Assessment of genetic diversity among Indian populations of *Cuscuta reflexa* based on ITS sequences of nrDNA

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Internal transcribed spacer (ITS) region of nuclear ribosomal DNA was amplified from 30 populations of *Cuscuta reflexa* Roxb. collected from nine different geographical locations of India. Analysis of nucleotide sequences reflects the polymorphism among the populations and differentiated them regionally. Maximum Likelihood Analysis (MLA) conducted on the ITS datasets, revealed that clade showed 100% bootstrap support (together) and subclade showed grouping according to their geographic locations in all the sampled populations except the samples collected from West Bengal and Assam. Thus, such results showed the distribution of *C. reflexa* in different geographical regions which might have evolved under reproductive isolation while the genetic exchange could be the reason in case of *C. reflexa's* populations distributed in the West Bengal and Assam.

Key words: *Cuscuta reflexa*, genetic diversity, internal transcribed spacer, nrDNA, maximum likelihood analysis.

INTRODUCTION

Cuscuta reflexa Roxb., which is commonly known as *Tukhm-e-Kasoos* (*dodder*), *Aftimoon* or *Kasoos* in the Unani Tibbi. The plant is immensely used in the Ayurvedic system of medicine to treat urination disorder, jaundice, muscle pain and coughs. The seeds are alterative, anthelmintic, carminative, and are widely used in the treatment of bilious disorders. The stems are also used in the treatment of bilious disorders and the whole plant is used as purgative. It is also used internally in treating protracted fevers and externally in the treatment of body pain and itchy skin. The juice of the plant is mixed with the juice of *Saccharum officinarum* which is used in the treatment of jaundice. 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; Khan et al., 2010). The methanol extract of *C. reflexa* exhibited anti-bacterial and free radical scavenging activity (Pal et al., 2006; Uddin et al., 2007). The petroleum ether extract of *C. reflexa* and its isolates are useful in the treatment of androgen-induced alopecia by inhibiting the enzyme 5-α-reductase activity possibly because of steroidal constituents (Pandit et al., 2008).

Phylogeny estimation is important for macro and micro evolutionary studies ever since cladistic and nucleotide sequencing techniques became available (Castelloe and Templeton, 1994; Vogler and DeSalle, 1994). During the last decade, some aspect of research has focused on phylogeny using various molecular markers of nuclear as well as organelle genomes (Avise, 1994). Internal transcribed spacer (ITS) sequences of nrDNA have been widely used for resolving phylogenetic relationships among closely related species of angiosperms (Pandey and Ali, 2006), molecular authentication of herbal materials (Zhang et al., 2007), genetic diversity assessment (Mondini et al., 2009), intra-specific variation study (Haque et al., 2009), and DNA barcoding (Zuo et al., 2010). The nrDNA region has frequent insertions/deletions which can be phylogenetically informative (Baldwin et al., 1995). The nuclear ribosomal transcriptional unit (NRTU) is comprised of 18, 5.8 and 28S genes, two ITS (ITS-1 and ITS-2), and an intergenic spacer (IGS) which found in hundreds to thousands of

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tandem copies and several are present within plant genomes. The conserved regions (18S and 28S genes) of NRTU are used to infer phylogenetic relationships at higher taxonomic levels, whereas the more rapidly evolving segments (ITS and IGS) are used for studies at the genic or population levels (Soltis and Soltis, 1998; Alvarez and Wendel, 2003). For over a decade, ITS sequences of NRTUs have been widely used to infer phylogenetic relationships, genetic diversity and to unravel evolution in a wide range of complexes in plants (Alvarez and Wendel, 2003; Baldwin and Markos, 1998; Baldwin et al., 1995; Hershkovitz et al., 1999; Kelch and Baldwin, 2003; Lee et al., 2002). Although, NRTUs are found in thousands of copies within a genome, intra-genomic diversity is generally low (Baldwin et al., 1995). This homogeneity among NRTUs is attributed to concerted evolution (Baldwin et al., 1995; Ainouche and Bayer, 1997), a process that acts through gene conversion and unequal crossing over. After transcription, the NRTU is processed to produce mature rRNAs which are key components of cytoplasmic ribosomes. Once the two copies meet, the fate of the polymorphism depends on genetic, reproductive and population-level factors: specifically, the number and location of ribosomal loci (on the same or different chromosomes), the occurrence of polyploidy and/or apomixes (Hershkovitz et al., 1999; Campbell et al., 1997; Buckler et al., 1997; Hughes et al., 2002), and the relative abundance of different ITS copies in the breeding populations (Feliner et al., 2004).

A perusal of literature (Costea et al., 2008, 2009; Costea and Stefanovic, 2009; Stefanovic et al., 2007; Khan et al., 2010) reveals that information on population structure and genetic variation of *C. reflexa* is lacking. Hence, the main objectives of the present study were to utilize the nucleotide data of the combined ITS1-5.8S-ITS2 sequences: (a) To reconstruct the phylogeny of Indian populations of *C. reflexa*, (b) To determine whether geographical differentiation occurs among regions, (c) To evaluate the degree of differentiation among *C. reflexa* populations from India and its relationship with geographical distribution.

MATERIALS AND METHODS

Plant materials

The stem material of *C. reflexa* was collected from natural habitat during plant explorations in different geographical region of India in July, 2007 (Figure 1). Two accessions of *C. japonica* (DQ211588, DQ924571) were selected as out group based on previous phylogenetic analyses of *Cuscuta* to serve as successively more distant out groups. The out group sequences were retrieved from NCBI GenBank (www.ncbi.nlm.nih.gov) and included in the analysis.

DNA extraction

The collected stem of *C. reflexa* was dried in silica gel prior to DNA extraction. Total genomic DNA was extracted with modified CTAB method (Khan et al., 2007).

Amplification of ITS region

ITS sequences of nrDNA were amplified using primers of White et al. (1990). ITS1 (Forward 5'-GTCCACTGAACCTTATCATTTAG-3') and ITS4 (Reverse 5'-TCCTCCGCTTATTGATATGC-3') with the polymerase chain reaction (PCR) using the AccuPower HF PCR PreMix (Bioneer, Daejeon, South Korea) in 20 µl volumes containing 2 µl of 10X buffer, 300 µM dNTPs, 1 µl of a 10 pM solution of each primer, 1 unit of HF DNA polymerase. One round of amplification consisting of denaturation at 94°C for 5 min. followed by 40 cycles of denaturation at 94°C for 1 min., annealing at 49°C for 1 min. and extension at 72°C for 1 min. with a final extension step of 72°C for 5 min. The PCR products were purified with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea) prior to sequencing.

DNA sequencing

The purified fragments were directly sequenced using dye terminator chemistry following the manufacturer's protocol. The sequencing reaction was performed in a 10 µl final volume with the BigDye Terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems). Cycle sequencing was conducted using same primers used in amplification and BigDye vers. 3 reagents and an ABI PRISM 3100 DNA Analyzer (Perkin-Elmer, Applied Biosystems). Cycling conditions included an initial denaturing set at 94°C for 5 min., followed by 30 cycles of 96°C for 10 s., 50°C for 5 s, and 60°C for 4 min. Sequenced product was precipitated with 17 µl of deionized sterile water, 3µl of 3 M sodium acetate, and 70 µl of 95% ethanol. Polyacrylamide gel electrophoresis was conducted with long ranger single packs (FMC BioProducts) and an ABI 3100 automated DNA sequencer (Perkin-Elmer, Applied Biosystems). Each sample was sequenced in the sense and antisense direction and analyzed with ABI sequence navigator software (Perkin-Elmer/Applied Biosystems). Nucleotide sequences of both DNA strands were obtained and compared to ensure accuracy.

Sequence alignment

Sequence alignments were performed using ClustalX version 1.81 (Thompson et al., 1997) and subsequently adjusted manually using BioEdit (Hall, 1999). Insertion-deletions (Indels) were scored as single characters when we had confidence in positional homology. The boundaries between the ITS1, 5.8S, and ITS2 were determined by comparisons with earlier published sequences available in NCBI GenBank. Gaps were treated as missing data in phylogenetic analyses. All sequences generated in the present study were deposited in GenBank and GenBank accession numbers are included in Table 1.

Sequence diversity and phylogenetic reconstruction

Nucleotide polymorphism, as measured by θw (Watterson, 1975) and diversity, as measured by π (Nei, 1978) were calculated using software DnaSP v4.5 (Rozas and Rozas, 1999). Analysis of molecular variance (AMOVA) was performed using software GenAlEx 6.1 (Peakall and Smouse, 2006) to assess genotypic variations across all the populations studied. This analysis, apart from partitioning of total genetic variation into within-group and among-group variation components, also provided a measure of intergroup genetic distance as proportion of the total variation

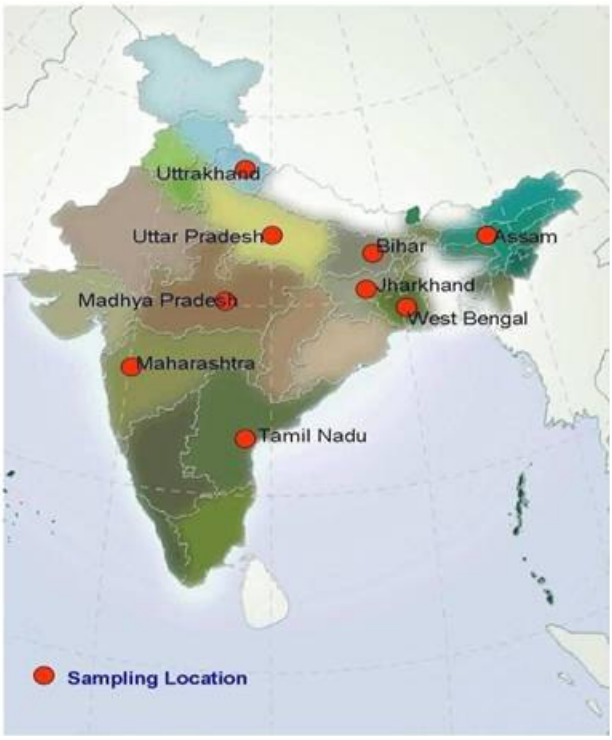

Figure 1. Geographical locations of *Cuscuta reflexa* in India.

residing between populations.

The significance of the analysis was tested using 999 random permutations. The maximum parsimony and maximum likelihood analysis of aligned sequences were performed using MEGA5 (Tamura et al., 2007).

RESULTS AND DISCUSSION

Nucleotide sequences and intra-specific divergence

The ITS sequences of nrDNA regions from 30 individuals of *C. reflexa* were analyzed. The amplified region of ITS1-5.8S-ITS2 in *C. reflexa* varied between 575-576 bases (57-58% GC content) with out group spacer lengths 568 bp (58% GC content). Data matrix has a total number of 606 characters of which invariable (monomorphic) sites (499), variable (polymorphic) sites (47) (total number of mutations, 49) and parsimony informative sites (47).

INDELs were necessary to align the sequences which ranged from 1 to 3 bp and the overall transition/transversion bias (*R*) was found 0.931. The substitution probabilities are given in Table 2. Polymorphism was observed among the populations [π = 0.01541, θw = 0.01195 (0.00428) and total variance 3.014], where π and θw refer to nucleotide diversity according to Nei and Li (1979) and Watterson's parameter (Watterson, 1975) respectively.

Figures in parenthesis denote standard deviation. Variability within the NRTU usually depends upon number of gene copies, rates of mutation, concerted evolution, number and chromosomal location of NRTU clusters, and proportion of sexual and asexual reproduction (Dover et al., 1993). Polymorphism may arise when concerted evolution is not fast enough to homogenize repeats in face of high rates of mutation (Appels and Honeycutt, 1986) or by loss of sexual recombination (Campbell et al., 1997).

AMOVA was used to partition the genetic diversity of *C. reflexa* and tested whether there was any hierarchy of ITS sequence variation among individuals (Table 3). The genetic differentiation between the populations is high (ΦST = 0.1994). Nei (1978) classified *G*ST > 0.15 as high, ΦST and *G*ST both denote fixation index and are comparable.

Phylogenetic reconstruction

The evolutionary history was inferred using the maximum parsimony method (Eck and Dayhoff, 1966). The parsimony analysis (100 bootstrap replicates) of the entire ITS region resulted in 352 maximally parsimonious trees (MPTs) with a total length of 52 (consistency index is 0.9423, the retention index is 0.9822, and the composite index is 0.9255). The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 3 (Felsenstein, 1985; Nei and Kumar, 2000) in which the initial trees were obtained with the random addition of sequences (10 replicates). The codon positions included in the parsimony analysis were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. All positions containing gaps and missing data which were eliminated from the dataset (complete deletion option). There were a total of 545 positions in the final dataset, out of which 47 were parsimony informative. Phylogenetic analysis was also inferred using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-1075.1249) is shown in Figure 2. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search are obtained automatically as follows: when the number of common sites is < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Substitution pattern and rates were estimated under the Kimura (1980) 2-parameter model (Kimura, 1980). The nucleotide frequencies are A = 20.86%, T/U = 22.43%, C = 27.55%, and G = 29.16%. For estimating ML values, a user-specified topology was used. The maximum log likelihood for this computation was -373.916. The estimated value of the shape parameter for the discrete gamma distribution was 0.2635. Substitution pattern and

Table 1. Sampling location of *C. reflexa* and GenBank accession number.

Geographic location	Population code	GenBank accession number
Bihar	CrefBH1	HQ728491
	CrefBH2	HQ728492
	CrefBH3	HQ728493
Jharkhand	CrefJK1	HQ728494
	CrefJK2	HQ728495
	CrefJK3	HQ728496
West Bengal	CrefWB1	HQ728501
	CrefWB2	HQ728502
	CrefWB3	HQ728503
Madhya Pradesh	CrefMP1	HQ728515
	CrefMP2	HQ728516
	CrefMP3	HQ728517
Tamil Nadu	CrefTN1	HQ728518
	CrefTN2	HQ728519
	CrefTN3	HQ728520
Assam	CrefAS1	HQ728504
	CrefAS2	HQ728505
	CrefAS3	HQ728506
Maharashtra	CrefMH1	HQ728511
	CrefMH2	HQ728512
	CrefMH3	HQ728513
	CrefMH4	HQ728514
Uttar Pradesh	CrefUP1	HQ728497
	CrefUP2	HQ728498
	CrefUP3	HQ728499
	CrefUP4	HQ728500
Uttarakhand	CrefUK1	HQ728507
	CrefUK2	HQ728508
	CrefUK3	HQ728509
	CrefUK4	HQ728510

rates were estimated under the Tamura-Nei (1993) model (+Gamma) (Tamura and Nei, 1993). A discrete gamma distribution was used to model evolutionary rate differences among the sites (5 categories, [+G]). Mean evolutionary rates in these categories were 0.00, 0.03, 0.21, 0.83, and 3.92 substitutions per site. All the tree resulted from maximum likelihood analysis reveals that all the sampled population of *C. reflexa* grouped together (100% bootstrap support), with two major clade (i) samples of *C. reflexa* collected from Bihar (India), Tamil Nadu (India), West Bengal (India) and Assam (India)- (57% bootstrap support), and (ii) the samples collected from Jharkhand (India), Maharashtra (India), Uttrakhand (India), Uttar Pradesh (India) and Madhya Pradesh (India)- (62% bootstrap support). Further, in each major clade, the samples collected from different geographical regions of Bihar, Jharkhand, Uttar Pradesh, Madhya Pradesh, Maharashtra, Uttrakhand and Tamil Nadu grouped according to their geographic locations {Bihar (71% bootstrap support), Jharkhand (87% bootstrap support), Uttar Pradesh (100% bootstrap support), Madhya Pradesh (96% bootstrap support), Maharashtra

Table 2. Maximum composite likelihood estimate of the pattern of nucleotide substitution.

	A	T	C	G
A	-	6.19	8.05	10.99
T	5.57	-	14.47	8.15
C	5.57	11.11	-	8.15
G	7.51	6.19	8.05	-

Each entry shows the probability of substitution from one base (row) to another base (column). Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics. The nucleotide frequencies are 0.199 (A), 0.221 (T/U), 0.288 (C), and 0.292 (G). The transition/transversion rate ratios are k_1 = 1.349 (purines) and k_2 = 1.797 (pyrimidines).

Table 3. Hierarchical analysis of molecular variance (AMOVA) within/among *C. reflexa* populations.

Source of variation	d.f.	SSD	Estimated variance	Total variance (%)	p-value
Among population	8	4.000	0.000	0	
Within population	21	10.500	0.500	100	1.000
Total	29	14.500	0.500		

d.f.: degrees of freedom, SSD: sum of squared deviations, p-value: the probability of having a more extreme variance component than the observed.

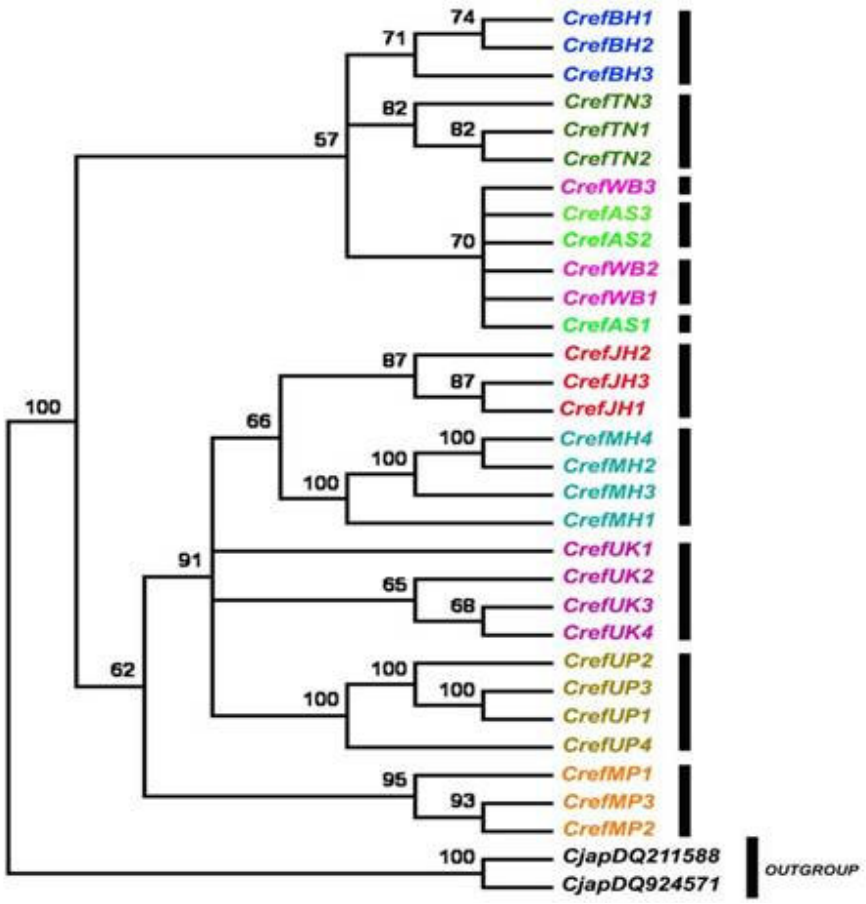

Figure 2. Evolutionary relationships of *C. reflexa* inferred using ML method implemented in MEGA5. Numbers on the branches indicate bootstrap support under 100 bootstrap replicates. The scale bar indicates relative length of the branch.

(100% bootstrap support), Uttrakhand (65% bootstrap support) and Tamil Nadu (82% bootstrap support)}, except, the samples collected from West Bengal and Assam did not grouped separately and were found to be mixed (70% bootstrap support). It is also interesting to note that one out of four accessions collected from Uttarakhand shows polytomic relationships among subclade (91% bootstrap support) in which accessions collected from the Jharkhand, Maharashtra, and Uttar Pradesh cluster together. Thus, the result of present analysis based on ITS sequences of nuclear ribosomal DNA clearly reveals that population of *C. reflexa* distributed in different geographical regions of India has evolved under reproductive isolation and it also seems that the genetic exchange might have taken place among the populations of *C. reflexa* distributed in the West Bengal and Assam.

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