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Full Length Research Paper

Assessment of genetic diversity among fenugreek (*Trigonella foenum-graecum* L.), using RAPD molecular markers

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Plant *Trigonella foenum-graecum* or fenugreek belongs to family Papilionaceae. Two taxonomically Trigonella species and 61 accession were analyzed with 18 random primers to evaluate genetic diversity and species relation. TFG and TC have been used for study, among these cultivars there is almost no or very little intraspecific difference in morphological manifestations however TFG and TC elite cultivars. Total genomic DNA was extracted by CTAB method with some modification. PCR amplification was carried out by using master cycler gradient thermal cycler. From total 18 primers screened across all species and accession scoring 141 bands of which 74 were polymorphic. On average 7.7 bands per primer were scored. The percentage of polymorphic band ranged from 66% to 100% with an average of 52.85%. Only the amplified DNA fragment ranging in size between 200 to 1000 bps was used for statistical analyses. Cluster analysis based on the presence or absence of band was performed by Jaccard's similarity coefficient, based on unweighted pair group method with arithemetic averages (UPGMA). Genetic similarity ranged between 0.66 to 0.90, indicating a moderate to high genetic variability. The highest similarity coefficient was detected between accession DA-30, VR-76, VR-67, DA- 03 and DM-37, and the lowest in accession TFG sp. and TG sp., respectively. The dendrogram revealed two main clusters. Each cluster was divided into subgroup. This investigation showed that RAPD marker is a useful tool for evaluation of genetic diversity and relationship amongst different *Trigonella* species.

Key words: *Trigonella foenum-graecum*, RAPD, varieties, primer, genetic diversity.

INTRODUCTION

Fenugreek (*Trigonella foenum-graecum* L.) is a common leguminous plant used as condiments and medicinal purposes (www. ANAGEN.Net). Since older time it is being prevailed to be used as potent antidiabetic (Puri, 2002), digestive, antipyretic, lactogogue, hypolipidemic, antioxidant and so many other purposes (Bhatti, 1996). Among spices it is a common crop plant. South Africa, Austria, Australia, Europe and Asian continents are major producer of Fenugreek. About 70 species of this plant are reported all over world. Area wise distribution of fenugreek germplasm is very vast. About 200 distinguished varieties of fenugreek are distributed in India belonging to Deshi and Champa elite varieties. In India, Rajasthan and M.P are major states of fenugreek production. The production of fenugreek seeds in 1996 to 1997 was about 40,000 tonnes and 38,062 tonnes in M.P and Rajasthan respectively covering more than 75,000 hectare land. Since 1988 to 2000 more than 237 articles of international repute have been published on Fenugreek (Agricola data base). The diversity analysis using the physiolological parameters showed grouped of *T. foenum graecum* cultivars presented significant correlation with the geographic fenugreek distribution

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(Marzaugui, 2009). Researchers are trying to produce improved varieties by conventional breeding (McCormic, 1998) and genetic engineering techniques (Das, 1997; Ahmad and Acharya, 1999). The *Trigonella* species large scale production and the development of better varieties are restricted by the lack of information about their genetic diversity, inter-and intra specific variability and genetic relationship among their species (Marzaugui et al., 2009). RAPD markers have been useful in evaluation of genetic diversity and markers assisted selection offers a great opportunity and effectiveness in selecting valuable plant genotypes (Young, 2002; Harris, 1999; Karp, 1998). Among PCR based markers RAPD markers are most useful because of low cost, speed and no need of radioactivity (Mohammadi and Prasanna, 2003; Williams and Kubelic, 1990). It is also used plant population genetic study (Rana and Bhat, 2002), phylogeny, gene tagging, gene mapping, (Hoque et al., 2002; Naghia et al., 2002) assessing genetic variations and identifying hybrids (Jug et al., 2004). Accessions undertaken for the study have been collected area wise from different locations of Uttaranchal state (Kumaon region) and grown in soil at defence agricultural research laboratory (DRDO) Pithoragarh for the breeding purpose. Objective of the study was to prepare distinguished genetic finger prints and correlate their genetic similarity and dissimilarity to exploit the technique for standard and successful breeding plant.

MATERIALS AND METHODS

Collection and maintenance of varieties

Representative set of 61 accession of two species of Fenugreek, group 1 of Fenugreek (*T. foenum-graecum)* with 59 accessions and group 2 Kasuri methi (*Trigonella corniculata*) with 2 accessions from different location of Uttaranchal, India (Table 1), were used for assessment of diversity. The accessions were selected randomly, which were maintained at Defence Agricultural Research Laboratory (DRDO), Pithoragarh, and Uttaranchal, India. These accessions have also been submitted to the gene bank of National Bureau of Plant Genetic Resources (NBPGR) New Delhi.

Extraction of genomic DNA

Total genomic DNA was extracted by CTAB method with some modification (Doyle and Doyle, 1990). Fresh young leaves from nursery raised plant individual genotype/ accession progeny were collected in ice box. Five gram leaf tissue was ground to fine powder in liquid nitrogen with a mortar and pestle. Leaf powder was then transferred into 50 ml polypropylene centrifuged tube containing 15 ml prewarmed (65°C) DNA buffer (2% CTAB, 20mM EDTA, 1.4 mM NaCl, 100 mM Tris cl pH 8.0) with 0.6 vol. of ß-mercaptoethanol and 2% PVP, tubes were shaken and incubated at 65°C for 60 min. Equal vol. of chloroform: Isoamyl alcohols (24:1) have been added and tubes were shaken end to end for 10 min to make emulsion, then centrifuged at 15,000 rpm for 10 min. 15 ml supernatant have been collected and subjected to RNAse treatment. Then DNA was precipitated using 0.6 vol chilled isopropanol, tubes were shaken end to end until DNA fibers appeared, centrifuged at 8,000 rpm, 4°C for 10 min. Pellets were washed with washing sol. (70% ethanol+10 mM ammonium acetate) and were dissolved into 1.0 ml TE buffer after drying. Ammonium acetate 7.5 mM (0.5 vol.) has been added and kept at -20°C for 30 min, centrifuged at 8000 rpm, 4°C for 20 min and supernatants have been collected into new tubes. DNA was precipitated using 4 to 5 ml absolute ethanol and pellets were dissolved in 1.0 ml TE buffer. Quantification and purity measurements of DNA have been performed by using UV spectrophotometer (Ultrospec-4000) and also analyzing the DNA on 0.8% agarose gel alongside diluted uncut λ DNA as standard.

DNA amplification

PCR amplification was carried out by using master cycler gradient thermal cycler (Eppendorf). Amplification was carried out in 50 µl reaction volume containing 1x Taq polymerase buffer (finnzyme), 1.5 mM magnesium chloride 4.5 µl, 200 µM each dNTP (Finnzyme), 20 µM primer, 1 unit of the Taq DNA polymerase enzyme (Finnzyme) and 50 ng of template DNA. Thermal cycler with an initial denaturation at 94°C for 3 min. followed by 42 cycles. Each cycle consisted of denaturation at 94°C for 45 sec., primer annealing at 42°C for 1 min, extension at 72°C for 3 min, with final extension at 72°C for 8 min. PCR products were separated on 1.5% agrose gel in 1x TBE buffer using ethidium bromide staining. The size of amplified fragments was determined by using size standard (3 kb DNA ladder Finnzyme). DNA fragment were visualized under U.V light and photographed using VSD Image master (Pharmacia Biotech). To test the reproducibility of the RAPD markers. The reactions were repeated at least twice.

Statistical analysis

Amplicons were scored as discrete variables, using 1 for the presence of bands and 0 for the absence of bands. Jaccard similarity coefficient based similarity matrix has been prepared by using the formula, (Jaccard, 1908). The Presence and absence banding pattern was submitted to NT-SYS-PC based software (SIMINT) to prepare similarity matrix. UPGMA (Unweighted Pair-group Method of Arithmetic average Analysis) have been performed by SHAN which uses similarity matrix of SIMQUAL as input file (Rohlf, 1975). Phylogenetic tree based on similarity matrix have been viewed by using graphics (TREEPLOT).

RESULTS AND DISCUSSION

Sixty one cultivars as represented in Table 1, belonging to two elite groups, 58 members of Methi and two members of Kasuri Methi. Total 141 amplicons were obtained with 18 primers. Highest number of bands was 12 as found in OPA-12 and lowest number of bands was 4 in OPW-9 with average of 7.7 bands per primer. The sizes of the amplified fragments range from 150-2 kb. DNA polymorphism as revealed by three of the highly polymorphic primers is depicted in (Figure 1). Of the 141 band scored, 74 (52.48%) were polymorphic and rest were monomorphic. The range of polymorphism was 33.33% (with 3 primers) to 100% (OPF-5) (Table 2). A total of three primers detected in this study which produce 4 unique alleles in five genotype (Table 3). These primers can be utilized as intervarietal molecular

Table 1. Varieties and their respective accession numbers.

Accession no	Name of varieties	Accession no	Name of varieties
1	DMM-49	32	DARL-140
2	VRS-VEE-1071	33	VDVSS-155
3	DARL-SS-68	34	VDVSS-74
4	DARL-SS-216	35	VDVSS-159
5	VRS-VFE-1167	36	DMRS-161
6	DARL-SS-103	37	VV-204
7	DMRS-237	38	DARL-SS-330
8	VRE-VFE-1992	39	VV-229
9	VDVSS-174	40	DMRS-256
10	VRB-VFE-1476	41	VRS-VFE-1056
11	DARL-SS-150	42	DARL-VP-1400
12	DARL-SS-145	43	DARL-VP-1383
13	DMRS-107	44	DARL-VP-1369
14	DARL-SS-44	45	DARL-VP-1363
15	KJ-186	46	DARL-VP-1357
16	VDVSS-123	47	DARL-VP-1352
17	DARL-VK-588	48	RKS-RAA-156
18	DARL-SS-36	49	DARL-VP-1191
19	DMRS-78	50	VR-VFC-2188
20	DMRS-72	51	VRVFE-2204
21	DARL-SS-211	52	VR-VFE-2207
22	KHH-654	53	VR-VFE-2326
23	DARL-SS-168	54	LOCAL ALMORA
24	VRB-VFE-2016	55	KASHMIRI
25	KHH-603	56	LOCAL PITHORAGARH
26	VRB-VFE-1791	57	SPECIAL
27	DMM-56	58	KASURI
28	DMM-520	59	KASTURI
29	KHH-185	60	DESHI
30	VV-254	61	LOCAL
31	DARL-SS-413		

marker to distinguish one or a few genotype from the rest of genotype. The pair wise comparisons of the RAPD profile based on both shared and unique amplification products to generate a similarity matrix. Similarity indices estimated on the basis of 18 primers ranged from 0.66 to 0.909. In similarity co-efficient values suggests that *T. foenum-graecum* germplasma collection represent a genetically diverse population and this might be attributed to a high level of cross pollination in this species (Dangi, 2004). The diversity revealed by RAPD is in agreement with the conclusion that out breeding plant species retains consideration variability (Gupta et al., 2008; Rana and Bhat, 2002). The genetic variance with in population was favored by genetic system of the species like gene flow, out breeding, mutation high genetic load etc. (Hoque et al., 2002; Matyas, 1996). Dendrogram was prepared by using Jaccard similarity coefficient NTSYS-pc based software SIMINT (Figure 2). Dendrogram showed two major clusters, smaller one having 2 varieties of Kasuri Methi which shared 99.05 similarity coefficients and larger cluster can be divided into 14 different sub-clusters. DARL-VP-1352 (Acc. No. 47) and DARL-SS-211 (Acc. No. 21) were separated into individuals. DMM-49, VRS-VEE-1071 and DMM-56 were closely related and shared a single cluster which was different from other 55 varieties. Deshi variety and Local Pithoragarh were closely related to each other. In separate Dendrogram of primers OPA-12, OPF-5, OPW-9, OPW-16, OPX-17, OPA-12 and OPW-2; Kas-1, Kas-2 shared 0.66, 0.8, 0.78, 0.80, 0.857, 0.667 and 0.909 similarity coefficient respectively. The highest value of similarity coefficient (0.92) was detected between DA-30, VR-76, VR-67 and DA-03. The lowest value of similarity coefficient (0.23) was evident between TFG and TC species respectively; it showed 2 distinguished clusters of TFG and TC cultivars. TFG cluster showed branching at

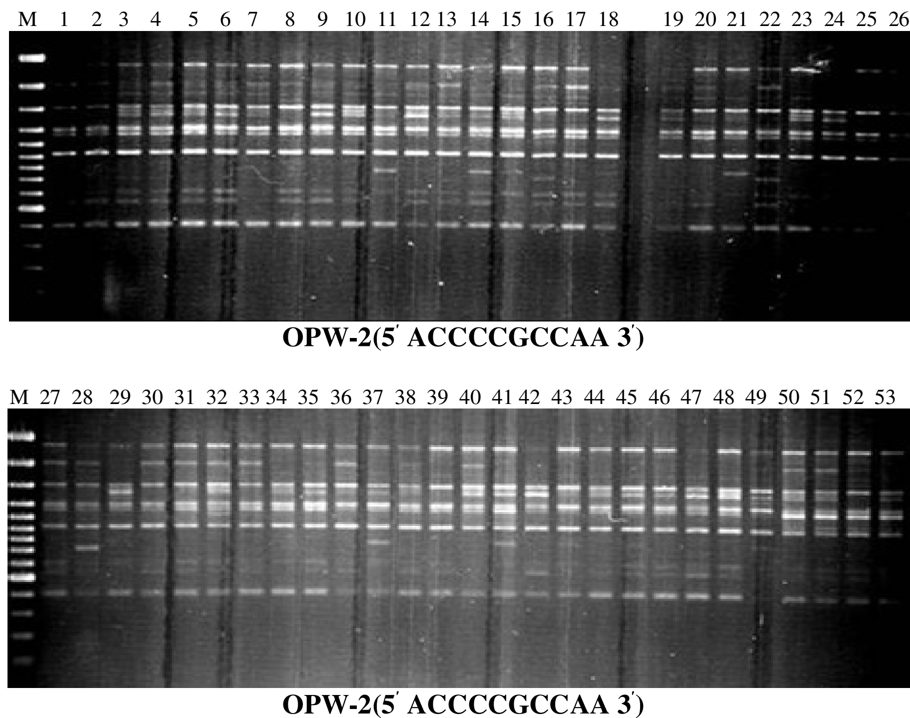

Figure 1. DNA Fingerprinting of 61 accession of *T. foenum-graecum* using different RAPD Primers: OPW-2, OPF-5, OPA-5.

Table 2. Primers with their respective number of bands and % polymorphism.

Primer	Sequence	Total no of bands	Total polymorphic bands	Polymorphism %
OPA-5	5`AGGGGTCTTG3`	10	4	40.00
OPA-12	5`TCGGCGATAG3`	6	2	33.33
OPA-13	5`CAGCACCCAC3`	5	3	60
OPA-18	5`AGGTGACCGT3`	12	8	66.60
OPD-7	5`TTGGCACGGG3`	11	4	33.36
OPD-13	5`GGGGTGACGA3`	10	5	50
OPD-15	5`CATCCGTGCT3`	7	4	57.10
OPF-5	5`CCGAATTCCC3`	5	5	100
OPM-5	5`GGGAACGTGT3`	6	2	33.30
OPW-2	5`ACCCCGCCAA3`	11	7	63.63
OPW-4	5`CAGAAGCGGA3`	10	4	40
OPW-5	5`GGCGGATAAG3`	8	3	37.50
OPW-8	5`GACTGCCTCT3`	7	4	57.10
OPW-9	5`GTGACCGAGT3`	4	3	75
OPW-16	5`CAGCCTACCA3`	5	2	40
OPX-8	5`CAGGGGTGGA3`	9	5	55.55
OPX-3	5`TGGCGCAGTG3	7	4	57.10
OPX-17	5`GACACGGAAC3`	7	4	57.10
		140		mean=54.14

0.77 similarity coefficient which separated DA-40 from remaining TFG varieties. DA-40 has been placed into a separate cluster in the dendrogram of OPW-2 which shared 0.69 similarity coefficient with another TFG sp. The larger TFG cluster again divided into two major groups in which DM-49, VR-71 and DM-20 shared

Table 3. Primer capable of amplifying Unique alleles from different genotype of TFG and TC.

No.	Primer	Total band	No. of unique alleles	Allele size (Kbp)
1	OPW-2	11	1	2
2	OPF-5	5	1	1.08
3	OPA-5	10	2	2 and 1.3

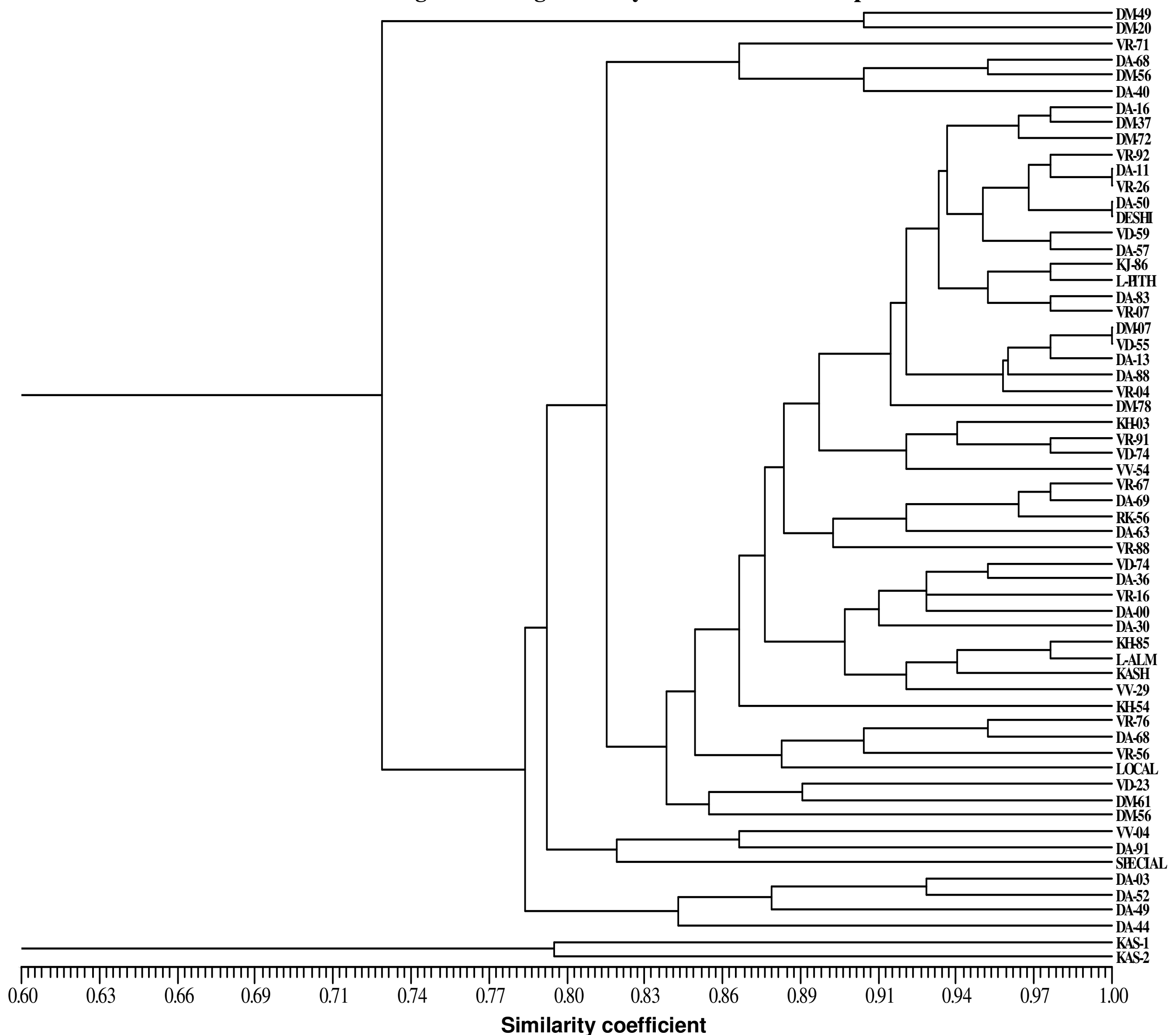

Figure 2. Dendrogram showing genetic relationship among various *T. foenum-graecum* genotypes.

similarity coefficient of 0.775 with other TFG spp. In this cluster DM-20 shared 0.864 similarity coefficient with VR-71 and DM-49. Combined dendrogram prepared by OPA-12, OPF-5, OPW-9, OPW-16, OPX-17, OPA-12 and opw-12 showed; that DA-11 and VR-35, DA-50 and DESHI, DM-07 and VD-55 were the same cultivars. This dendrogram is more or less similar to the combined dendrogram which, have been prepared by using

18 primers. So, these 7 primers are enough good to evaluate the diversity and intergenic or intragenic phylogenetic relationship among different TFG and TC cultivars. Cluster analysis reflected their geographical distribution. Ecological and geographical differentiations are two important factors, which influence breeding and sampling strategies of crops (Mc Cormick, 1998). Which further help in understanding the population structure, variation in genetic diversity with in the species is usually related with geographic range, mode of reproduction, mating system, seed dispersal and fecundity (Pasquet, 2002). The genetic diversity detected in the present studies may be due to all these prevalent background factors as the genotype of *T. foenum-graecum*. Studied are widely distributed in different ecogeographical region. In conclusion, molecular markers allowed us to estimate the overall genetic diversity in *T. foenum-graecum* and simultaneously revealed molecular based genetic relationship. The results of the present study showed that *T. foenum graecum* germplam with in India constitute a broad genetic base. From the clustering pattern and genetic relationship obtained using RAPD markers, breeders can identify the diverse genotype from different cluster and employ them in their future breeding programme.

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