



Assessment of genetic diversity in fenugreek (*Trigonella foenum-graecum*) genotypes using morphological and molecular markers

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ABSTRACT

Fenugreek (*Trigonella foenum-graecum*) is one of the most important seed spices possessing aromatic and medicinal properties. Commonly conventional methods based over phenotypic variability are being used by breeders for identification of superior germplasm which are laborious and time consuming. A study was carried out at Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Maharana Pratap University Agriculture and Technology, Udaipur, during 2014–16 to assess the genetic diversity among 20 diverse fenugreek genotypes using morphological and molecular markers for the identification of superior entries. Mean squares due to genotypes were highly significant and wide mean range performance was observed for plant height, pods per plant, test weight, biological yield per plot and harvest index. RAPD analysis was carried out with 25 primers and 21 primers produced 76 bands, in which 37 were polymorphic bands. Average polymorphism was 48.60% and Jaccard's Similarity Coefficient lies between 0.71 to 0.99. From the results based over morphological and molecular studies it could be concluded that 20 fenugreek entries were classified in 2 clusters through morphological characterization while the same entries were more precisely classified in 5 clusters through RAPD analysis. Superior fenugreek entries were identified for seed yield and its components. Based on the results promising and diverse fenugreek entries will be useful for further breeding programmes.

Key words: Diversity, Fenugreek, Molecular marker, RAPD, Seed yield

Fenugreek (*Trigonella foenum-graecum* L.) is a dicotyledonous, annual autogamous crop belonging to Fabaceae family. It originated from Mediterranean region (Duke *et al.* 1981). It is well known for aromatic, condiments and medicinal properties. Fenugreek seeds are used as dietary proteins and have antipyretic, antidiabetic digestive, lactagogue, hypolipidemic, and cholesterol reducing properties (Srinivasan 2014). It is grown in India, Egypt, Pakistan, France, England, Argentina and North African Countries (Mc *et al.* 2009). In India, it is extensively grown in the tropical and subtropical regions of India. Rajasthan, MP and Gujarat are major producer and contribute 88%

production of the country.

Availability of germplasm pool is essential to develop a new desirable traits or creating allelic variation. In *Trigonella* 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). Recently researchers are using molecular markers which have become important tools in the studies of genetic diversity (Sharma *et al.* 2015). Among the PCR based molecular markers, random amplified polymorphic DNA (RAPD) technique has been extensively used to assess the genetic diversity, phylogeny, gene tagging, gene mapping and to detect genetic variations as well as to identify hybrids (Fracaro *et al.* 2005, Sundaram and Purwar 2011, Sharma *et al.* 2018). Limited previous studies were addressed on genetic diversity within populations of fenugreek using ISSR, RAPD and AFLP techniques (Dangi *et al.* 2004, Kumar *et al.* 2012).

Systematic evaluation and quantification of the variability from the present study will serve as one step towards providing accurate genetic information for breeding programmes of fenugreek improvement. Thus, the aim of this study was to investigate diversity of fenugreek accessions using morphological and RAPD markers and to characterize

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relationship among fenugreek populations from different geographical regions.

MATERIALS AND METHODS

Plant material: The pure nucleus seed of 20 diverse fenugreek genotypes originated from different geographical sources (Table 1) were procured from Department of Genetics and Plant Breeding, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur. The genotypes were sown during winters, 2014–16 in randomized block design with three replications and maintained in the uniform agro-climatic conditions at the instructional farm of the Rajasthan College of Agriculture, MPUAT, Udaipur (24.5568° N, 73.7153° E), (Rajasthan), India. The observations were recorded for seedling vigour, days to 50% flowering, plant height, number of branches per plant, number of pods per plant, pod length, number of seeds per pod, test weight, biological yield per plot, seed yield per plot and harvest index were recorded. The fresh 22 days old young leaves were collected for DNA isolation and molecular diversity analysis.

Extraction of genomic DNA: Total genomic DNA was extracted by CTAB method with some modifications (Doyle and Doyle 1990). Quantification and purity measurements of DNA were performed by using UV spectrophotometer (Ultrospec-4000) and also analyzed 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). Master mix volume was 20 μ l containing 1xTaq polymerase buffer, 2.5 mM $MgCl_2$, 2.0 μ l, 200 μ M each dNTP, 200 μ M primer, 1 unit of the Taq DNA polymerase enzyme (Bangalore genei) and 50 ng of template DNA. Thermal cycler with an initial denaturation at 94°C for 5 min followed by 44 cycles consisted of denaturation at 94°C for 60 second, primer annealing at 37°C for 1 min, extension at 72°C for 2 min, with final extension at 72°C for 7 min.

Data analysis: Total number of band within each line and number of polymorphic bands were noted. Each DNA fragment amplified by a given primer was considered as a unit character and the RAPD fragments were scored as a binary variable (1) for presence and (0) for absence of each of the primer accession combination. The presence or absence of polymorphic and non-polymorphic bands was scored in a binary data matrix. Cluster data for the genetic distance was carried out using UPGMA clustering method. To check the morphological behaviour with the genetic traits clustering based on morphological character using WARD'S method and its cross validation were also done. All the statistical analysis was performed using NTSYS2.2, SAS 9.2 and JMP Genomics 4 software.

RESULTS AND DISCUSSION

Fenugreek is one of the most important seed spice crops. It is consumed in multiple ways and has high medicinal value. Looking to its importance in state's economy, the present study was carried out using 20 diverse cultivars of fenugreek to characterize them through morphological and molecular (RAPD) markers. This will prove a clear-cut and precise output to assess the diversity for further speed breeding of the crop.

Morphological characterization is the first step in the description and classification of germplasm. A sound knowledge of various morphological traits in the breeding material helps in classification, identification, naming and documentation of the genotypes in a crop. The mean squares due to genotypes were highly significant for all the traits thereby indicating substantial amount of genetic variability among the genotypes (Table 2).

The range and mean values of all the 10 characters are presented in Table 3 revealed narrow mean values for seeds per pod, branches per plant and pod length. Moderate mean range was exhibited for character such as 50% flowering, while most of the characters, viz. plant height, pods per plant, test weight, seed yield, biological yield and harvest index had wide mean range. Similar results were also observed in fenugreek for different characters, viz. pods per plant, 1000 seed weight, pod length and seeds per plant by Kumar *et al.* (2003) and Meena *et al.* (2011).

Ward's cluster analysis: Ward's hierarchical cluster analysis was carried out for 10 morphological characters in fenugreek. It was used to measure genetic distance between 20 fenugreek genotypes. Cluster analysis grouped the genotypes into two clusters. Cluster I and II were apart

Table 1 Twenty diverse fenugreek genotypes with their origins

Code No.	Genotypes	Origin/Sources
V1	NS 2006-1	SK RAU, Jobner (Rajasthan)
V2	NS 2006-2	SK RAU, Jobner (Rajasthan)
V3	NS 2006-3	SK RAU, Jobner (Rajasthan)
V4	NS 2006-4	SK RAU, Jobner (Rajasthan)
V5	NS 2006-5	SK RAU, Jobner (Rajasthan)
V6	NS 2006-6	SK RAU, Jobner (Rajasthan)
V7	NS 2006-7	SK RAU, Jobner (Rajasthan)
V8	UM 134	SD Agriculture University, Jagudan (Gujarat)
V9	UM 152	Coimbatore (Tamil Nadu)
V10	UM 163	Coimbatore (Tamil Nadu)
V11	UM 189	Coimbatore (Tamil Nadu)
V12	UM 202	SD Agriculture University, Jagudan (Gujarat)
V13	UM 353	Bhawani Mandi, Jhalawar
V14	UM 354	Bhawani Mandi, Jhalawar
V15	JFG 244	SD Agriculture University, Jagudan (Gujarat)
V16	RMT-303	Mutant of RMT-1
V17	RMT-305	Mutant of RMT-1
V18	RMT-351	Mutant of RMT-1
V19	RMT-1	SK RAU, Jobner (Rajasthan)
V20	RMT-143	SK RAU, Jobner (Rajasthan)

Table 2 Analysis of variance of fenugreek genotypes for 10 characters

Character	Replication	Genotype	Error
Degree of Freedom	[2]	[19]	[38]
Days to 50% flowering	2.51	36.35**	1.30
Plant height	1.01	25.41**	2.69
Branches per plant	0.09	0.83**	0.03
Pods per plant	2.53	81.31**	1.58
Pod length	0.73	1.20	0.07
Seeds per pod	0.23	4.80**	0.07
Test weight	2.61	3.63**	1.81
Seed yield	0.008	0.08**	0.005
Biological yield	0.019	1.66**	0.030
Harvest index	4.25	32.15**	7.40

**Values significant at 1%

at 25 rescaled values.

Cluster I included 16 genotypes. This cluster was further sub divided into two sub-clusters, viz. A and B at 9

rescaled values. Sub-cluster A included nine genotypes, viz. V12 (UM-202), V17 (RMt-305), V18 (RMt-351), V3 (NS 2006-3), V9 (UM 152), V5 (NS 2006-5), V10 (UM 163), V-13 (UM 353) and VI (NS 2006-1). Where genotypes V12 (Um 202), V17 (RMT-305), V18 (RMT-351), V3 (NS 2006-3), V9 (UM 152), V5 (NS 2006-5), V10 (UM 163), V13 (UM 353) were 3 unit apart from VI (NS 2006-1). Characters like plant height, pods length, seeds per pod, test weight, biological yield, seed yield and harvest index did not exhibit much variation in these genotypes, whereas variation was recorded for days to 50% flowering, pods per plant and branches per plant.

Sub-cluster B included seven genotypes, viz. V4 (NS 2006-4), V19 (RMt-1), V14 (UM 354), V15 (JFG 244), V11 (UM 189), V2 (NS 2006-2) and V20 (RMT-143) where V4 (NS 2006-4) and V-19 (RMT-1) where 2 unit away from V14 (Um 354), V15 (JFG 244), V11 (UM 189) and V2 (NS 2006-2). Genotypes V20 (RMt-143) were 6 unit apart from rest of the genotypes. Days to 50% flowering, plant height, branches per plant, pods length and test weight were almost at par in all these genotypes, whereas significant variation were observed for pods per plant, seeds per pod,

Table 3 Mean performance of 20 fenugreek genotypes for 10 different characters

Genotype	Days to 50% flowering	Plant height (cm)	Branches/ plant	Pods/ plant	Pod length (cm)	Seeds/ pod	Test weight (g)	Biological yield/plot (kg)	Seed yield/plot (kg)	Harvest index (%)
NS 2006-1	47.00	59.67	5.63	35.20	10.53	16.60	13.27	4.67	1.45	31.07
NS 2006-2	46.00	61.33	5.50	38.53	11.07	17.00	13.20	3.87	1.30	33.53
NS 2006-3	40.00	59.67	6.13	30.30	11.33	14.40	12.80	4.04	1.21	29.67
NS 2006-4	49.00	61.33	5.83	31.70	10.47	16.07	12.23	3.23	1.20	37.11
NS 2006-5	40.67	61.00	6.10	30.27	10.57	15.67	12.60	4.33	1.34	30.85
NS 2006-6	48.67	63.67	5.67	41.47	11.27	19.70	14.18	5.93	1.65	27.87
NS 2006-7	41.00	63.67	6.60	39.87	10.63	17.20	13.27	5.03	1.38	27.48
UM 134	47.67	63.33	6.37	37.20	10.83	16.37	15.33	5.20	1.32	25.32
UM 152	40.33	64.00	5.67	43.43	10.63	17.73	13.20	4.17	1.53	36.80
UM 163	49.00	62.00	5.77	36.33	9.43	16.23	13.23	4.30	1.39	32.33
UM 189	48.00	65.67	7.00	41.33	10.57	16.63	12.37	3.57	1.39	38.88
UM 202	48.33	65.33	5.70	43.43	11.27	17.10	12.27	4.10	1.37	33.33
UM 353	48.33	61.33	5.40	34.20	11.50	18.20	13.33	4.40	1.45	32.95
UM 354	46.33	63.33	6.43	30.53	9.33	18.43	12.33	3.60	1.07	29.63
JFG 244	46.67	63.00	7.03	31.47	9.67	17.57	14.20	3.67	1.20	32.73
RMt- 303	48.33	70.67	5.73	32.03	10.37	17.67	12.40	5.23	1.67	31.85
RMt- 305	41.67	66.00	5.87	44.67	10.37	18.40	13.23	4.10	1.38	33.74
RMt- 351	47.33	63.33	5.43	42.93	10.40	18.37	9.90	4.13	1.34	32.42
RMt- 1	40.67	69.67	6.23	30.70	9.53	17.27	12.77	3.40	1.10	32.35
RMt- 143	41.67	61.33	6.97	31.47	10.23	15.30	13.53	2.90	1.07	36.78
Grand mean	45.53	63.47	6.05	36.35	10.50	17.08	13.01	4.20	1.34	32.33
Range	40-49	59.7-70.7	5.4-7.0	30.2-44.7	9.3-11.5	14.4-19.7	9.9-15.3	2.90-5.93	1.07-1.67	25.3-38.8
SEm±	0.66	0.95	0.10	0.72	NS	0.16	0.77	0.1	0.04	1.57
CD (5 %)	1.83	2.62	0.28	2.01	NS	0.14	2.15	462.27	192.84	4.35
CV (%)	2.52	2.58	2.91	1.38	2.01	1.64	10.32	4.13	5.39	8.51

biological yield, seed yield and harvest index. Cluster II included genotypes, viz. V7 (NS 2006-7), V8 (UM 134), V16 (RMT-303) and V6 (NS 2006-6). Genotypes, viz. V7 (NS 2006-7) and V8 (UM 134) were 2 unit apart from V16 (RMT-303) while V6 (NS 2006-7) was 7 unit away from V7 (NS 2006-7), V8 (UM 134) and V16 (RMT-303). These genotypes showed significant variation for pod per plant, seeds per pod, biological yield, seed yield and harvest index only. These revealed that the accessions were mostly identical and less diversity. The results were in conformation with the observation of Talebi *et al.* (2008) who studied genetic relationship among 36 accessions of chickpea.

Molecular analysis: RAPD markers were used for analysis of genetic diversity from the isolated DNA. The concentration of isolated DNA ranged between 0.40 µg/µl (RMT-305 and RMT-351) to 2.65 µg/µl (NS 2206-6 and UM 354). The quality of DNA was determined by calculating the ratio between O.D. at 260 nm and O.D. at 280 nm which ranged from 1.78 (Um-353) to 2.05 (RMT-305) indicating fairly high quality of isolated DNA.

All the 20 diverse varieties of fenugreek cultivars were examined for DNA polymorphism using 25 RAPD primers. Out of 25 primers 21 primers produced amplification, whereas 4 primers did not show any amplification. Again out of 21, only 15 primers showed variable degree of polymorphism ranging from 25% (RPI 11) to 100% (RPI 4, RPI 6, RPI 7, RPI 15, RPI 18 and RPI 20), whereas RPI 5 RPI 13, RPI 16, RPI 19 and RPI 21 primers did not show any polymorphism (Table 4).

The RAPD primer RPI 15 and RPI 20 generated 3 bands with 100% polymorphism and another primers, viz. RPI 4, RPI 6, RPI 7 and RPI 18 gave bands and showed 100% polymorphism. Whereas 50% polymorphism were showed by three primers, viz. RPI 10 and RPI 23 respectively. Total 76 bands were generated out of which only 37 polymorphic bands. The average number of bands per primer was found to be 3.6. The average numbers of polymorphic bands per primer were 1.76 overall polymorphism was found to be 48.68 per cent. Similar results were observed by Raina *et al.* (2001) in peanut cultivars and obtained 42.7% polymorphism.

Genetic relationship: The banding pattern generated and polymorphic patterns were used to calculate the genetic similarity using method Jaccard's Coefficient Analysis. The similarity coefficient matrix generated for the primers was subjected to algorithm UPGMA and dendrogram was generated using NTSYS-pc 2.02 programme (Rohlf 1997). The similarity coefficient for different genotypes was in the range of 0.71 to 0.99. The average similarity across all the genotypes was found to be 0.85 indicating a high level of genetic similarity among the genotypes. This maximum similarity coefficient (0.99) was observed between V19 (RMT-1) and V20 (RMT-143) followed by V6 (NS2006-6) and V8 (UM 134), V13 (UM353) and V16 (RMT-303) showed similarity of 0.97. The minimum similarity coefficient (0.71) was observed between V4 (NS 2006-4) and V7 (NS 2006-7), V4 (NS 2006-4) and V7 (NS

Table 4 Polymorphism information of RAPD primers analyzed

Primer code	Total No. of band (a)	Total No. of polymorphic band (b)	Polymorphism % (b/a × 100)
RP 1	NA	NA	NA
RP 2	5	4	80
RP 3	NA	NA	NA
RP 4	4	4	100
RP 5	4	0	0
RP 6	4	4	100
RP 7	4	4	100
RP 8	3	0	0
RP 9	4	2	50
RP 10	2	1	50
RP 11	4	1	25
RP 12	3	1	33
RP 13	6	0	0
RP 14	3	1	33
RP 15	3	3	100
RP 16	2	0	0
RP 17	3	2	66
RP 18	4	4	100
RP 19	5	0	0
RP 20	3	3	100
RP 21	2	0	0
RP 22	6	2	33
RP 23	2	1	50
RP 24	NA	NA	NA
RP 25	NA	NA	NA
Total	76	37	48.68
Average	3.6	1.76	-

NA: Not amplified

2006-7). The results obtained were in conformity with the earlier report by Dangi *et al.* (2004) selected 17 accession of fenugreek to study molecular diversity using RAPD assay using 100 primers for initial screening in *T. foenum-graecum* of which 22 primers generated polymorphic patterns revealing 70.12% polymorphism.

RAPD dendrogram: Dendrogram was constructed using similarity matrix value as determined from RAPD data depicted the relationship among the genotypes of fenugreek. The dendrogram generated on the basis of Jaccard's Similarity Coefficient, clearly indicated five main clusters (Fig 1).

Cluster I was the major cluster and it included five genotypes, viz. VI (NS 2006-1), V5 (NS 2005-5), VII (UM 189) V17 (RMT-305) and V10 (Um 163) at a similarity coefficient of 0.914. This cluster was divided into three sub-clusters A, B and C. Sub-cluster A included VI (NS 2006-1) at Similarity Coefficient of 0.914, whereas sub-cluster B consisted three genotypes V5 (NS 2006-5), V11 (UM 189)

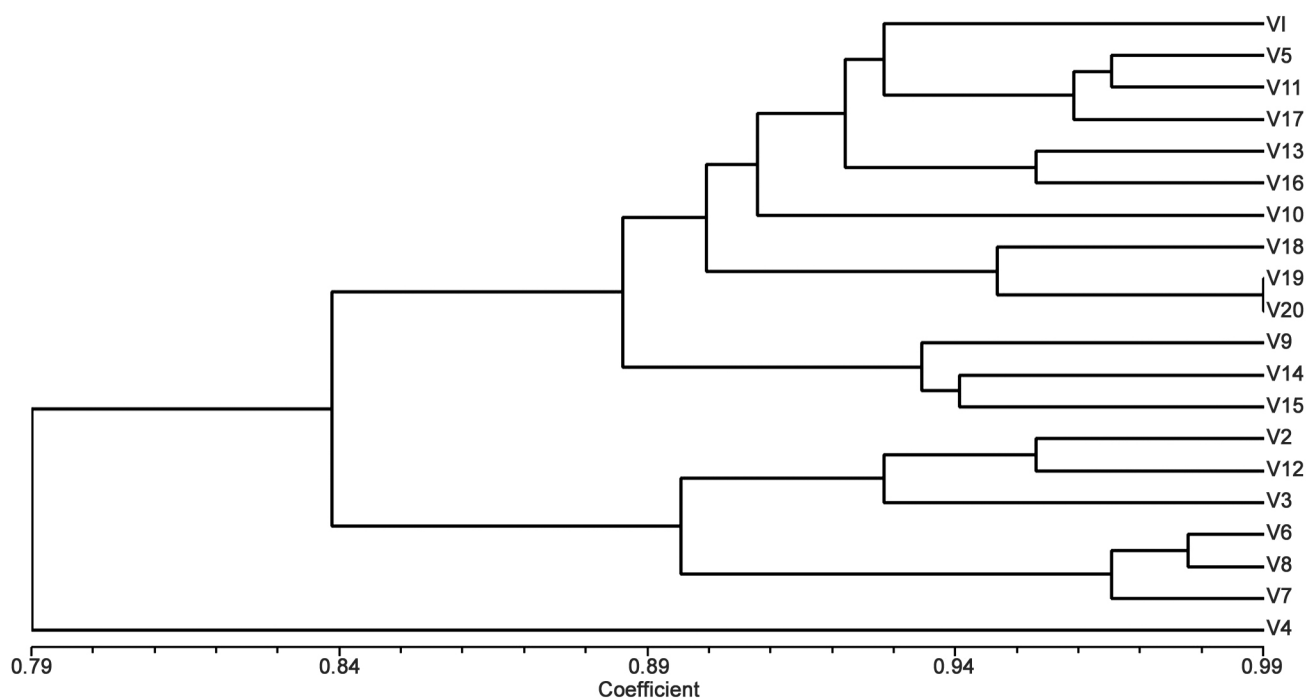


Fig 1 Dendrogram generated for 20 fenugreek genotypes using UPGMA cluster analysis based on RAPD data.

and V-17 (RMt-305) at a similarity coefficient of 0.951. Sub-cluster C include only V10 (UM 163) at similarity coefficient of 0.90. Cluster II included three genotypes V18 (RMT-351), V-19 (RMT-1) and V-20 (RMT-143) at similarity coefficient of 0.93. Cluster III included five genotypes V9 (UM 152), V15 (JFG 244), V14 (UM 354), V13 (UM 353) and V16 (RMt-303) at similarity coefficient of 0.91. Cluster IV included six genotypes V2 (NS 2006-2), V12 (UM 202), V3 (NS 2006-3), V6 (NS 2006-6), V8 (UM 134) and V7 (NS 2006-7) at a similarity coefficient of 0.88. While V cluster include only one genotype, i.e. V4 (NS 2006-4) where related to cluster IV at similarity coefficient of 75. Similarly, Chowdhury *et al.* (2001) also studied the genetic diversity of 47 accession of soybean characterized by means of agro morphological trait and RAPD marker.

Principal Component Analysis (PCA): Two and three dimensional principal component analysis based on RAPD data showed similar clustering pattern of 20 genotypes as evident from cluster tree analysis. Most of the genotypes were in five main clusters and several sub clusters. On the basis of morphological and RAPD analysis it can be concluded that entries NS 2006-1, UM 353 and JFG 244 appeared superior, genetically diverse and promising for seed yield and its component characters, viz. days to 50% flowering, seeds per pod, test weight and harvest index. Further superior entries were also identified for seed yield (NS 2006-5, UM 189, UM 163 and RMt-305). These entries also originated from different geographical sources; hence these could be gainfully utilized in breeding programmes.

In conclusion, results indicated presence of moderate genetic variability among the elite fenugreek genotypes through morphological and RAPD based molecular markers.

Results derived from this study would be highly useful in fenugreek breeding programmes as molecular markers can be successfully utilized for determining genetic diversity precisely.

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