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SSR Based Genetic Diversity Analysis in Fenugreek (*Trigonella foenum-graecum* L.) Genotypes

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ABSTRACT

Background: Fenugreek (*Trigonella foenum-graecum* L.) is an important seed spice crop widely grown all over the world. In India, the state of Rajasthan is known for fenugreek production and productivity in the world. A concerted assessment of genetic variability among the germplasm accession is essential for breeding new superior varieties. Molecular markers such as AFLP, RAPD, ISSR, SSR, SCAR, SCoT, SRAP have become for the characterization of the germplasm rapidly and accurately. The present study aimed to characterize 20 elite fenugreek genotypes using simple sequence repeat (SSR) markers to assess the existing genetic diversity of this medicinal crop.

Methods: The present study was carried out at the Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, Rajasthan, India. Total genomic DNA was isolated from old leaves using the CTAB method (Doyle and Doyle, 1990). Further, PCR based genetic diversity was analyzed with using 50 SSR primer pairs. Dendrogram was constructed using NTSYSpc version 2.2 and clustering of the genotypes was done.

Result: Twenty genotypes of fenugreek were assessed for genetic diversity analysis using SSR markers. Out of 50 markers 43 primer pairs produced 130 alleles with an average of 84.60% polymorphism. Jaccard's similarity coefficient lied between and 0.39 to 0.82. Based on UPGMA clustering, a dendrogram consisting of five main clusters was generated with wide variability among the studied genotypes. These diverse genotypes so identified could be gainfully utilized in the fenugreek breeding programme.

Key words: Dendrogram, Diversity analysis, Fenugreek, Molecular marker SSR.

INTRODUCTION

Fenugreek (*Trigonella foenum-graecum* L.) is an annual, herbaceous, diploid leguminous crop, belongs to the subfamily Papilionaceae of Fabaceae. It is one of the oldest crop known for aromatic, condiments and medicinal purposes. It originated in the Mediterranean region (Duke *et al.,* 1981). It is a dual-purpose crop used as a vegetable as well as a spice for human consumption (Bromfield *et al.,* 2001; Tufail *et al*. 2020). Fenugreek leaves are a rich source of calcium, iron, carotene and vitamins A and D while seeds are used as dietary proteins. It has anti-diabetes, antipyretic, antidiabetic digestive, lactogogue, hypolipidemic and cholesterol-reducing properties as a potent antioxidant (Srinivasan, 2014). The crop also acts as a soil stabilizer and thereby increases its fertility. Fenugreek is largely grown in India, Egypt, Ethiopia, Pakistan, United Kingdom and North African countries in varied agro-climatic and growth conditions (Maloo *et al*., 2020). Rajasthan, Madhya Pradesh and Gujarat are major producers and contribute ~88% production of India.

Availability of germplasm pool is essential for developing new desirable traits. Assessment of existing genetic diversity in the germplasm is essential to select new desirable traits. Conventionally morphological traits are used to assess the existing variability but these are highly influenced by the environment. The approach of molecular fingerprinting is complementary to phenotypic measures in quantifying genetic changes because it shows variations in DNA that may not be morphologically expressed. Molecular markers are reliable tools to assess the genetic diversity in plants. The PCR based molecular markers are being extensively used in different crops to assess the genetic diversity rapidly (Tomar *et al*., 2014; Sharma *et al*., 2015; Nath *et al*., 2017 and Choudhary *et al*., 2019). These are also used for genetic, phylogeny, gene tagging, gene mapping studies and assessing genetic variations as well as identifying hybrids (Sharma *et al*., 2018) revealing variation at the DNA level with genetic polymorphism. Studies were made for analyzing genetic diversity within populations of fenugreek using molecular markers ISSR, RAPD and AFLP techniques (Kumar *et al*., 2012; Mamatha *et al*., 2017 and Maloo *et al*., 2020). However, limited reports are available related to

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simple sequence repeat (SSR) markers in fenugreek. Therefore, an attempt was made in this study to estimate the extent of polymorphism and inter-specific diversity in fenugreek *elite* accessions/ varieties using SSR markers to identity desirable genotypes which could be gainfully utilized in future breeding programmes.

MATERIALS AND METHODS

Plant material

The nucleus seed of twenty fenugreek genotypes/varieties originated from different geographical sources (Table 1) was procured from the Department of Genetics and Plant Breeding, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, India. The work was carried out at Department of Molecular Biology and Biotechnology Centre, RCA, MPUAT, Udaipur during 2017-2018. Genotypes were sown in pots. Fresh 22 days old young leaves were collected for DNA isolation and molecular diversity analysis.

DNA extraction and PCR amplification

Total genomic DNA was extracted from 22 days old young leaves by the CTAB method with some modification (Doyle and Doyle, 1990). Quantification and purity measurements of isolated DNA were performed by using UV Spectrophotometer (Ultrospec-4000) and also on 0.8% agarose gel alongside diluted uncut λ DNA as standard. PCR amplification was carried out in 20 µl reaction volume (1xTaq buffer, 2.5 mM, $MgCl_2$ 2.0 µl of 200 µM each dNTP, 1 unit of the Taq DNA polymerase, 1.0 µl of 200 µM primer: Bangalore genie and 50 ng of template DNA) in 200 µl PCR tubes. PCR programme for SSR initial denaturation at 94°C for 4 min, primer annealing at a specific temperature for 1 min, extension at 72°C for 2 min, with a final extension at 72°C for 8 min. Amplified PCR products were separated on 1.8% agarose gel in 1x TAE buffer using ethidium bromide with a 100 bp DNA ladder. DNA fragments were visualized under UV light and photographed using VSD Image master (Pharmacia Biotech). To test the reproducibility of the marker, the reaction was repeated at least twice.

Data analysis

Total number of alleles within each line and number of polymorphic alleles were noted. Each DNA fragment amplified by a given primer was considered as a unit character and SSR fragments were scored as a binary variable (1) for presence and (0) for the absence of each of the primer accession. The presence or absence of polymorphic and non-polymorphic alleles was scored in a binary data matrix. To study the genetic similarity and dissimilarity clustering of data was done. Cluster data for the genetic distance was carried out using the UPGMA clustering method. Statistical analysis was performed using NTSYS2.20f, SAS 9.2 and JMP Genomics 4 Software (Rohlf, 2004).

RESULTS AND DISCUSSION

Fenugreek is one of the most important spice crops largely grown in Rajasthan which is also termed as “Bowl of Fenugreek” and produces more than 77.5% of the country’s fenugreek. Looking its importance in the Indian economy, the present study was carried out using 20 fenugreek genotypes to characterize them through SSR molecular markers to record precise assessment of genetic diversity for further improvement.

Molecular markers offer plant geneticists and breeders a set of genetic tools that are abundant, non-deleterious, reliable since these are least affected by environmental fluctuation and heritable. In the present investigation, SSR markers were used for the analysis of genetic diversity. DNA was isolated from the young leaf through the CTAB method and analyzes the concentration which 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.

Table 1: Twenty diverse fenugreek genotypes/ varieties and their origin.

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

In this study 50, RM series SSR primers were screened and only 43 primers produced amplification. Twenty-five primers showed 100% polymorphism with an average of 84.60% polymorphism among the studied genotypes. Forty-three primers amplified a total of 130 alleles, out of which 110 were polymorphic. The average number of alleles per primer was 3.02, while the average numbers of polymorphic alleles per primer were 2.5 (Table 2). A high level of polymorphism was also observed by Selvaraj *et al*. (2011) in rice, Kumari *et al*. 2013 in *Amaranthus spp*., Mandal *et al*. (2018) in lentil, Suman *et al*. (2018) in mung bean.

Genetic relationship among fenugreek genotypes and cluster analysis

The similarity coefficient ranged from 0.39 to 0.82 with a maximum similarity coefficient (0.82) was observed between V8 (UM 134) and V9 (UM 152), followed by V1 (NS 2006-1) and V2 (NS 2006-2) which was 0.80. The minimum similarity coefficient (0.39) was observed between V16 (RMt-303) and V17 (RMt-305).

Cluster I which was the major cluster included three genotypes *viz.,* V1 (NS 2006-1), V2 (NS 2006-2) and V3 (NS 2006-3) at a similarity coefficient of 0.749 (Fig 1). This cluster was divided into two sub-clusters namely A and B. Sub-cluster A had two genotypes *viz.,* V1 (NS 2006-1) and V2 (NS 2006-2). Cluster II contained five genotypes *viz.,* V5 (NS 2006-5), V6 (NS 2006-6), V7 (NS 2006-7), V8 (UM 134) and V9 (UM 152) at a similarity coefficient of 0.719. This cluster was divided into two sub-clusters *viz*., C and D. Sub-cluster C included only V5 (NS 2006-5) at a similarity coefficient of 0.719, which sub-cluster D consisted of four genotypes *viz.,* V6 (NS 2006-6), V7 (NS 2006-7), V8 (UM 134) and V9 (UM 152) at a similarity coefficient of 0.762. Cluster III included four genotypes *viz.,* V11 (UM 189), V13 (UM 353), V12 (UM 202) and V15 (JFG 244) at a similarity coefficient of 0.642. This cluster was divided into two sub-clusters E and F. Sub-cluster E included one genotype *viz*.,V11 (UM 189) at a similarity coefficient of 0.642 whereas sub-cluster F consisted of three genotypes *viz.,* V13 (UM 353), V12 (UM 202) and V15 (JFG 244) at a similarity coefficient of 0.695. Cluster IV included five genotypes *viz.,* V14 (UM 354), V17 (RMt-303), V18 (RMt-351), V19 (Rmt-1), and V20 (RMt-143) at a similarity coefficient of 0.652. This cluster was divided into two sub-clusters G and H. Sub-cluster G was further sub divided into two sub groups G1 and G2. G1 included single *viz.,* V14 (UM 354) at a similarity coefficient of 0.715 whereas G2 consisted of two genotypes *viz.,* V17 (RMt-303) and V18 (RMt-351) at a similarity coefficient of 0.730. Cluster V included three fenugreek entries *viz.,* V4 (NS 2006-4), V10 (UM 163) and V16 (RMt-303) at a similarity coefficient of 0.508. This cluster was divided into two sub-clusters I and J. Sub-cluster I included one genotype *viz.,* V4 (NS 2006-4) at a similarity coefficient of 0.508 whereas the sub-cluster II consisted V10 (UM 163) and V16 (RMt-303) at a similarity coefficient of 0.571(Fig 1).

Table 2: Level of polymorphism obtained from SSR primers.

Primers code	Total no. of alleles (a)	Total no. of polymorphic alleles (b)	Polymorphism % (b/a × 100)
RM3	1	0	0
RM 4A	1	0	0
RM 4B	NA	NA	NA
RM 5	2	1	50
RM 6	2	1	50
RM 7	NA	NA	NA
RM 8	1	0	0
RM 9	4	4	100
RM 10	6	6	100
RM 11	1	0	0
RM 12	1	0	0
RM 13	4	4	100
RM 14	3	3	100
RM 16	NA	NA	NA
RM 17	1	0	0
RM 18	4	4	100
RM 19	4	4	100
RM 20A	2	2	100
RM 20B	4	4	100
RM 21	NA	NA	NA
RM 22	NA	NA	NA
RM 23	2	2	100
RM 24	6	6	100
RM 25	1	0	0
RM 26	1	0	0
RM 27	7	7	100
RM 29	3	3	100
RM 30	5	5	100
RM 31	3	3	100
RM 33	2	1	50
RM 34	5	5	100
RM 35	1	0	0
RM 36	4	4	100
RM 38	4	4	100
RM 39	3	3	100
RM 41	4	4	100
RM 42	6	6	100
RM 44	4	4	100
RM 47	4	4	100
RM 48	4	4	100
RM 49	NA	NA	NA
RM 50	2	0	0
RM 51	1	0	0
RM 52	NA	NA	NA
RM 53	3	1	34
RM 55	1	0	0
RM 60	1	0	0
RM 70	1	0	0
RM 71	6	6	100
RM 72	5	5	100
Total	130	110	84.60
Average	3.02	2.5	

RM- Rice microsatellites.
NA- Not amplified.

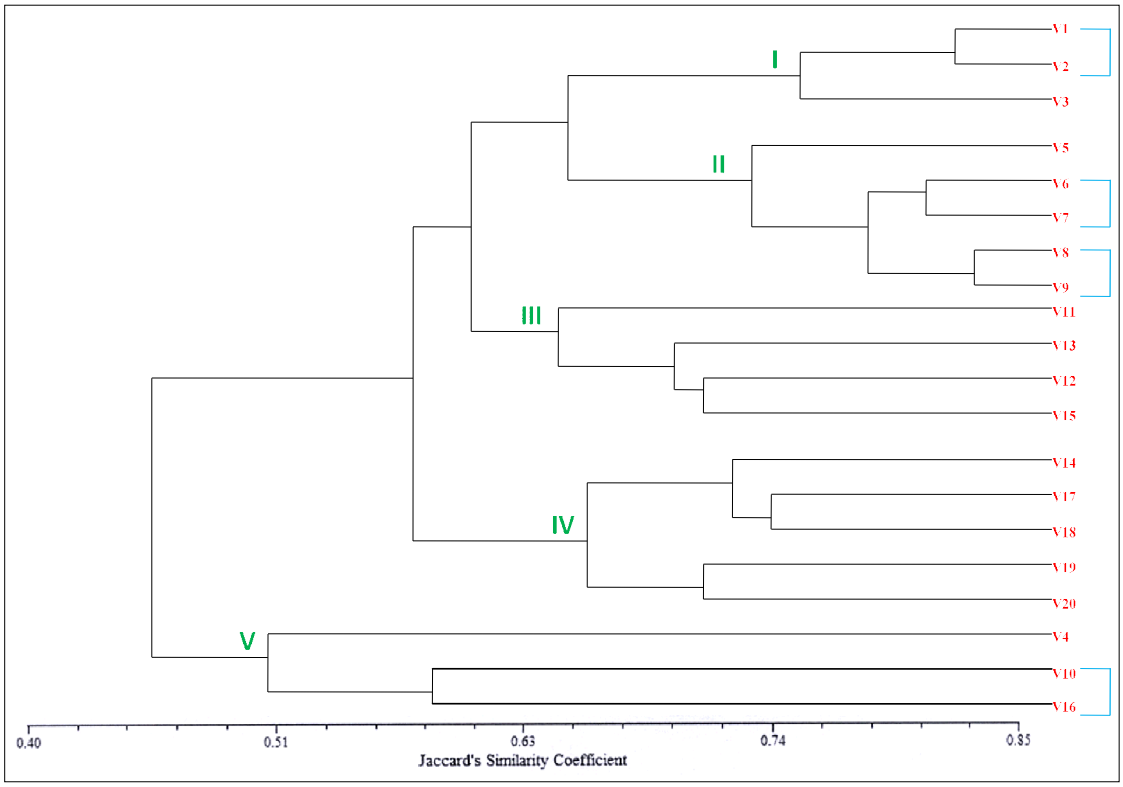

Fig 1: Dendrogram generated for 20 fenugreek genotypes using UPGMA cluster analysis based on SSR data.

CONCLUSION

In the present study, SSR markers were used to determine the genetic affinities between fenugreek genotypes at the DNA level. The results indicated that SSR markers could be effectively used in the determination of genetic relationships among fenugreek species. The dendrograms obtained through SSR showed classification of genotypes into 5 clusters reflecting moderate to high genotypic diversity in the fenugreek genotypes. It also indicated evidence for the relationship between fenugreek populations from different regions in India. The results further indicated that SSR markers can be precisely and effectively used in the determination of genetic diversity therefore diverse fenugreek genotypes could be gainfully utilized for future breeding programmes.

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