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ASSESSMENT OF GENETIC DIVERSITY USING SSR MARKERS IN FENUGREEK (*TRIGONELLA FOENUM-GRAECUM* L.)

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ABSTRACT

Fenugreek (*Trigonella foenum-graecum* L.) is an annual self-pollinated diploid species popularly grown by its vernacular name “methi”, belonging to the sub-family “Papilionaceae” of the family “Fabaceae” having chromosome number 2n=16 is grown majorly as a spice crop. The present investigation was carried out at 27 Fenugreek accessions, 40 random primers to evaluate genetic diversity and species relation. Total genomic DNA was extracted by CTAB method with some modification. PCR amplification was carried out by using master cycler gradient thermal cycler. A total of 40 SSR primers were used, of which 22 markers successfully amplified DNA from the 27 Fenugreek genotypes. The PIC values ranging from 0.32 to 0.48 (with an average of 0.42) indicate substantial diversity among the Fenugreek genotypes at the specific genetic loci targeted by the SSR markers. Cluster analysis based on the presence or absence of band was performed by Jaccard’s similarity coefficient, based on unweighted pair group method with arithmetic averages (UPGMA). The dendrogram revealed eight main clusters. Clusters were divided into subgroup. Cluster II and cluster VI contains maximum genotypes. This investigation showed that SSR marker is a useful tool for evaluation of genetic diversity and relationship amongst different *Trigonella* species.

Key words : *Trigonella foenum-graecum*, SSR, Varieties, Primer, Genetic diversity.

Introduction

Fenugreek (*Trigonella foenum-graecum* L.) is an annual self-pollinated diploid species popularly grown by its vernacular name “methi”, belonging to the sub-family “Papilionaceae” of the family “Fabaceae” having chromosome number 2n = 16 is grown majorly as a spice crop. The place of origin of fenugreek supposed to between Iran and North India (Smith, 1982). Fenugreek can be grown under a wide range of climatic conditions extending widely to warm temperate and tropical regions of the Mediterranean, Europe and Asia. India is a leading producer and consumer of fenugreek for its culinary uses and medicinal application. Fenugreek seeds and leaves are strongly aromatic and flavorful. The seeds are bitter in taste, but lose their bitterness if lightly roasted. They are rich in vitamins such as thiamin, folic acid, riboflavin, niacin, vitamins A, B6, C and are a rich storehouse of many minerals such as copper, potassium, calcium, iron, selenium, zinc, manganese and magnesium. Fenugreek leaves are a rich source of vitamin K as well. Fenugreek seeds are a rich source of trigonelline, lysine and l-tryptophan. The seeds also contain a large amount of saponins and fibers that may account for many of the health benefits of fenugreek like reduces cholesterol, regulates blood sugar and controls diabetes, enhances breast milk production, protects from cancer, maintains healthy testosterone levels, aids digestion, helps with weight loss and fenugreek’s use as a natural home remedy. It seed contains carbohydrates (48%), proteins (25.5%), mucilaginous matter (20.0%), fats (7.9%) and saponin (4.8%) (Rao and Sharma, 1987). The major fenugreek producing countries are India, Ethiopia, Egypt

and Turkey. India is one of the major producer and exporter of fenugreek. In India, the major fenugreek growing states are Rajasthan, Gujarat, Tamil Nadu, Uttar Pradesh, Haryana, Madhya Pradesh, Andhra Pradesh and Punjab. Rajasthan is considered as "fenugreek bowl" of the country and contributes about 90% to the country's production. India leads fenugreek production worldwide with an area of ~126 thousand ha, production of 182,170 tonnes and average productivity of 1,012 kg ha^{-1} as of 2019-20 (Nadeem *et al*., 2023). Knowledge of genetic diversity in a crop species is fundamental to its improvement. Evaluation of genetic diversity levels among adapted, elite germplasm can provide predictive estimates of genetic variation among segregating progeny for pure line. Genetic diversity can be identified using morphological, biochemical and molecular markers. But the morphology and biochemical markers of plant are influenced by environment and developmental stages of crop. In this context molecular markers act as best tool for evaluating genetic diversity of germplasm. Molecular markers are more reliable and these markers are not influenced by environment as well as the plant development stages and are also very less time consuming compared to the morphological evaluation. Among different polymerase chain reaction (PCR) based markers, Simple Sequence Repeats (SSRs) are more used as they detect high allelic diversity levels. The main goal of the study is to distinguish the germplasm of fenugreek and laying the foundations for purposive breeding programs to take full advantages of desirable genotypes at different geographical locations of the country.

Materials and Methods

Plant material

In present investigation, twenty- seven genotypes of fenugreek were collected from Department of Horticulture, J.N.K.V.V., Jabalpur and National Research Centre on Seed Spices, Ajmer, Rajasthan (Table 1). Field experiment was conducted on Vegetable Research Centre, Maharajpur, Department of Horticulture of Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur (M.P.), India. The Germplasm was sown in seed beds during second fortnight of October 2022 in Randomized Block Design (RBD) with a spacing of 30 cm × 10 cm and plot size of 3.3m × 2.2 m. The maximum temperature ranges between 24°C and 45°C and annual relative humidity of 80-90% in rainy season, whereas it was 60 to 70% in winter season and 30 to 40% in summers.

Extraction of genomic DNA

To make fine powder, about two grams of fresh young leaves were picked and crushed in liquid nitrogen

Table 1 : Names of the Fenugreek Genotypes used for the study.

S. no.	Name of genotype	Source of collection
1.	FGK- 139	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
2.	FGK-142	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
3.	FGK-143	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
4.	FGK-144	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
5.	FGK-145	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
6.	FGK-146	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
7.	FGK-147	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
8.	FGK-148	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
9.	FGK-149	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
10.	FGK-150	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
11.	FGK-151	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
12.	FGK-152	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
13.	FGK-153	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
14.	FGK-154	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
15.	FGK-155	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
16.	FGK-156	Department of Horticulture, JNKVV, Jabalpur, Madhya Pradesh
17.	AM-31	NRC Seed Spices, Ajmer, Rajasthan
18.	AM-91	NRC Seed Spices, Ajmer, Rajasthan
19.	AM-309	NRC Seed Spices, Ajmer, Rajasthan
20.	AFg-05	NRC Seed Spices, Ajmer, Rajasthan
21.	AM-115	NRC Seed Spices, Ajmer, Rajasthan
22.	AFg-03	NRC Seed Spices, Ajmer, Rajasthan
23.	AFg-04	NRC Seed Spices, Ajmer, Rajasthan
24.	AM-319	NRC Seed Spices, Ajmer, Rajasthan
25.	AM-45	NRC Seed Spices, Ajmer, Rajasthan
26.	AM-235	NRC Seed Spices, Ajmer, Rajasthan
27.	Pusa Kasturi	IARI, New Delhi

Fig. 1 : General view of Experimental Plot.

Fig. 2 : View of Fenugreek plant.

in a pre-cooled pestle and mortar. The crushed powder was transferred to 2ml sterile polypropylene tube that had been pre-warmed to 65°C and included 1ml of extraction buffer (CTAB, NaCl, 0.1 M tris HCl of pH 8.0, 0.5 M EDTA and β mercaptoethanol). The samples were incubated for 30 minutes in a water bath at 65°C, with tubes being mixed every 10 minutes. At room temperature, the mixture was centrifuged for 10 minutes at 10,000 rpm. The supernatant was transferred to new centrifuge tubes, which were then incubated in a water bath at 37°C for 15 minutes. A similar amount of chloroform: isoamyl alcohol (24:1) was added and the mixture was gently mixed for 5 minutes. For next 10 minutes, the mixture was centrifuges at 10,000 rpm. The supernatant was transferred to a new tube, and an equivalent volume of cold iso propanol was added, gently mixed by inverting 2-3 times and maintained at room temperature for 10 minutes to ensure appropriate precipitation. 1 ml cut tips were used to spool out precipitated DNA, which was then transferred to a 1.5 ml micro centrifuge tube. The supernatant was removed and the remaining DNA pellets were washed twice with 70% ethanol after spinning at 12000 rpm for 5 minutes. After drying at room temperature, the pellet was dissolved in 100 ìL of nuclease free water and stored at -20 degree Celsius for future use.

To assess the DNA's quality, a 0.8% agarose gel was employed. The presence of distinct, clear bands confirmed the absence of RNA and protein contamination. Furthermore, both the purity and quantity of the DNA were evaluated using a spectrophotometer (Hitachi U-1900®) measuring absorbance at wavelengths of 260 nm and 280 nm, respectively. These steps were crucial in verifying the integrity of the DNA samples for the study.

DNA amplification

Amplified DNA fragments were resolved by submerged horizontal agarose gel electrophoresis in 2.5 percent (w/v) agarose gel (for the PCR products of SSR markers) and visualized by staining with Ethidium bromide. Agarose solution was prepared in 1X TBE and ethidium bromide (10mg/ml) was added in the gel at a concentration of 2.5 µl per 100 ml of gel and then 34 mixed gently. It was poured in gel casting tray with appropriate comb with required well number and size. PCR products were mixed with loading dye, mixed and loaded into wells. The gel was run at voltage of 65 volt for 1 hour, which was then recorded in a gel documentation system.

Statistical analysis

Darwin software was used to observe data analysis. A UPGMA dendrogram was also created using the data produced by the cluster analysis. The polymorphism in SSR was recorded based on the presence or absence of the SSR bands in different varieties under investigation in the present study. All the varieties were scored for the presence and absence of the SSR bands. The data were entered into the binary matrix as discrete variables and this data matrix was subjected to further analysis. Genetic similarities among varieties were calculated based on the presence and absence of common bands. The genetic associations among varieties were analysed by calculating the similarity coefficient (Jaccard, 1908) for pair-wise comparisons based on the proportions of shared bands produced by primers.

Similarity coefficient = a/ (a+b+c)

Where,

A = Number of bands between Jth and Kth genotypes

B = Number of bands present in Jth genotype, but absent in K^{th} genotype

C = Number of bands absent in jth genotype but present in K^{th} genotype.

Similarity coefficients were used to construct UPGMA (Unweighted Pair Group Method with Average) to generate a dendrogram. Genetic similarity 49 values based on Jaccard s coefficients were converted into the genetic distance by subtracting the similarity index (matrix) value from unity. GD = 1- SI. The polymorphic percentage of the obtained bands was calculated by using the following formula, Polymorphism % = (Number of polymorphic bands / Total bands) × 100. In our molecular

analysis using SSR markers, alleles were designated based on fragment size; bands were scored as diallelic (1 = band present, 0 = band absent) and stored in an excel spreadsheet (Microsoft) file as well in NEXUS format. The agarose gel electrophoresis data was used to calculate the number of bands produced by each primer, the percent of bands shared by all varieties of Fennel, as well as the percent of bands, which were unique to only one variety. The coefficients of genetic similarity for all pair-wise comparisons were computed using Jaccard s coefficient, and then the distance matrix was subjected to cluster analysis by the unweighted paired group method using arithmetic average (UPGMA) to produce a dendrogram.

Results and Discussion

In this study, a total of 40 SSR primers were used, of which 22 markers successfully amplified DNA from the 27 Fenugreek genotypes (Table 2). The Polymorphic Information Content (PIC) values reflect the diversity at specific genetic loci. Higher PIC values indicate greater genetic diversity, suggesting that the selected markers can effectively discriminate among the genotypes. The PIC values ranging from 0.32 to 0.48 (with an average of 0.42) indicate substantial diversity among the Fenugreek genotypes at the specific genetic loci targeted by the SSR markers. Higher PIC values (0.48 for RM18, RM19 and Satt246) suggest significant genetic differentiation among these genotypes at those loci (Table 3).

Phylogenetic tree was constructed from genotyping data of selected polymorphic SSR markers using Darwin software on the basis of genetic distances. The genetic similarity among genotypes was estimated from the dissimilarity (distance) matrix generated from simple matching coefficient. The resulting dissimilarity matrix was further analysed using the unweighted pair-group method arithmetic average (UPGMA) clustering

Table 2 : Primers used in experiment.

S. no.	SSR Primer Code	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Tm (°C)
1.	RM 9	GGTGCCATTGTCGTCCTC	ACGGCCCTCATCACCTTC	52.24°C
2.	RM 13	TCCAACATGGCAAGAGAGAG	GGTGGCATTCGATTCCAG	56.30°C
3.	RM 18	TTCCCTCTCATGAGCTCCAT	GAGTGCCTGGCGCTGTAC	55.30°C
4.	RM 19	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA	52.35°C
5.	RM 20B	ATCTTGTCCCTGCAGGTCAT	GAAACAGAGGCACATTTCATTG	55.30°C
6.	RM 23	CATTGGAGTGGAGGCTGG	GTCAGGCTTCTGCCATTCTC	55.24°C
7.	RM 30	GGTTAGGCATCGTCACGG	TCACCTCACACGACACG	52.24°C
8.	RM 36	CAACTATGCACCATTGTGGC	GTACTCCACAAGACCGTACC	56.00°C
9.	RM 38	ACGAGCTCTCGATCAGCCTA	TCGGTCTCCATGTCCCAC	55.00°C
10.	RM 72	CCGGCGATAAAACAATGAG	GCATCGGTACTAACTAAGGG	54.51°C
11.	RM 21	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAC	53.35°C
12.	Sat_246	GCGCATGGTTTACAGATTACTTTA TTTTCT	GCGGCAATCATTTAAATTTATAAT GATATA	52.9°C
13.	Satt 448	GCGCTAAGGGCAATTTTATTTCAA	GCGCAGCCTGTTCAGTTTTTCTTTTGT	54.5°C
14.	Satt 686	ACGGAAAATAAATGAAACTAAGA	GCGCTATCAGATAGAGAAGCAGAAGAAT	55.0°C
15.	Satt 518	GCGCATATCAAATTGCATATAAAAATA	GCGCGAATATAAAATAAAAATGCTCA	54.2°C
16.	Satt 114	GGGTTATCCTCCCCAATA	ATATGGGATGATAAGGTGAAA	53.4°C
17.	Satt 042	GACTTAATTGCTTGCTATGA	GTGGTGCACACTCACTT	52.6°C
18.	Satt 236	GCGTGCTTCAAACCAACAAACAACTTA	GCGGTTTGCAGTACGTACCTAAAATAG	56.0°C
19.	Satt 119	TGTGCCAGTGTTGATAGTTA	CTGATCCCCAATAAATCTG	52.2°C
20.	Satt139	TATTATAAAAATCAATGCGAAAGG	CTTTTTAATAAGCCCAAATAATTACAT	54.5°C
21.	Satt 275	GCGGGATAATTGGTTTTACGAAAATGC	GCGCCTAATCACCTAAAAAAACGTTTA	56.0°C
22.	Satt 293	GCGCAGAAGGTTTGCATAAAAAGAAT	GCGGGCTAAAAAGTTGATGTAATGTG	54.9°C

Plate 1 : SSR profile of 27 fenugreek genotypes obtained with Satt 042.

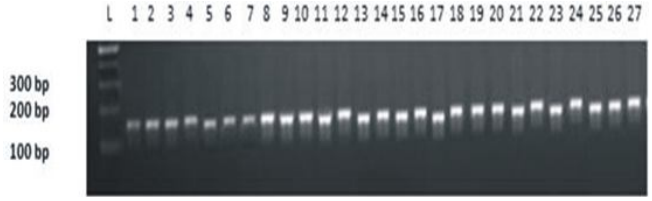

Plate 2 : SSR profile of 27 fenugreek genotypes obtained with Satt 119.

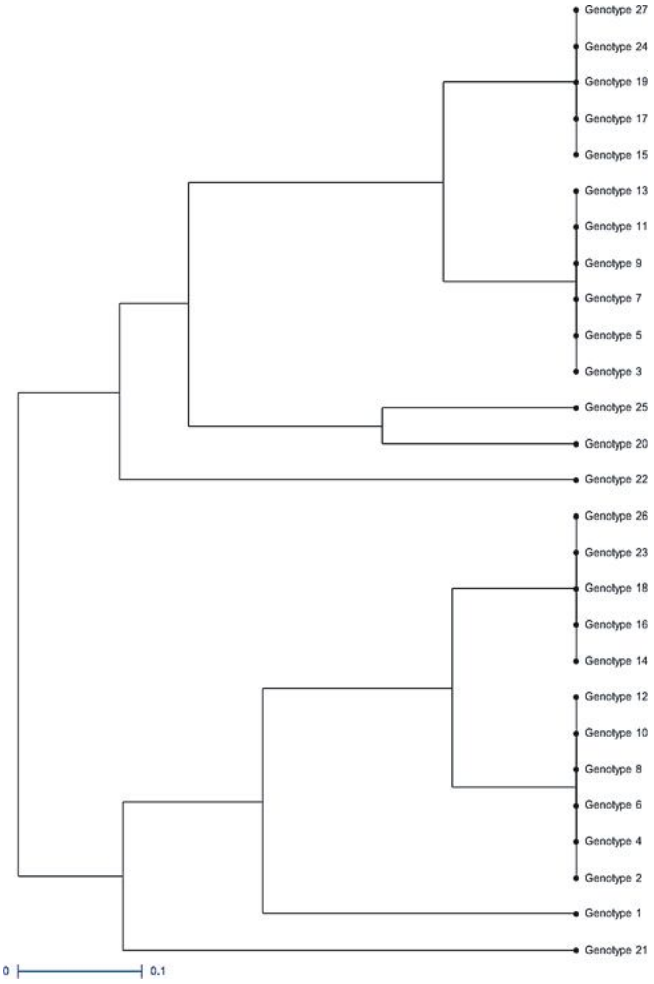

Fig. 3 : UPGMA Dendrogram.

algorithm for construction of a dendrogram. The UPGMA tree shows the genetic relationship between the fenugreek germplasm lines . All the 27 genotypes were grouped in 8 clusters. Cluster II and cluster VI contains maximum genotypes *i.e.*, six genotype in each cluster including FGK-153, FGK-151, FGK-149, FGK-147, FGK-145, FGK-143 in Cluster II and FGK-152, FGK-150, FGK-148, FGK-146, FGK-144, FGK-142 in cluster IV. Cluster

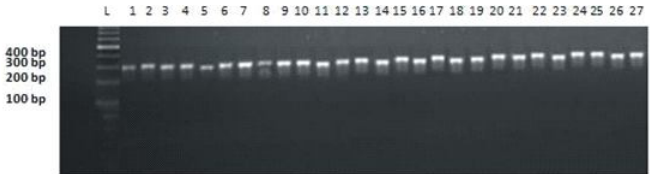

Plate 3 : SSR profile of 27 fenugreek genotypes obtained with Satt 518.

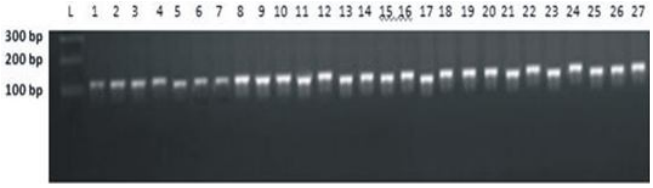

Plate 4 : SSR profile of 27 fenugreek genotypes obtained with Satt 448.

Table 3 : PIC value (Polymorphic Information Content).

Primer	No. of Alleles	PIC
RM 36	2	0.46
Satt 686	2	0.43
RM18	2	0.48
RM 72	2	0.42
Satt 518	4	0.46
Satt 236	2	0.34
RM 9	2	0.36
RM19	2	0.48
RM 20 B	2	0.44
RM 23	2	0.43
RM 21	2	0.43
RM13	2	0.35
RM 30	2	0.47
RM 38	2	0.41
Satt 448	3	0.32
Satt 042	3	0.42
Satt 246	2	0.48
Satt 114	2	0.39
Satt 119	2	0.45
Satt 139	2	0.37
Satt 275	2	0.38
Satt 293	3	0.42

I includes five genotypes Pusa Kasturi, Afg-04, AM-309, AM-31, FGK-155. Cluster V includes AM-45, Afg-03, AM-91, FGK-156, FGK154. Cluster III includes AM-319 and AM-235. Cluster IV contained AM-115. Cluster VII and cluster VIII includes FGK-139 and Afg-05, respectively. Notably, when comparing the cluster analysis results obtained from SSR primers with those derived from morphological data, slight disparities were observed. Maloo *et al.* (2023), Maloo *et al.* (2020), Sindhu *et al.* (2017), Randhawa *et al.* (2012), Kumar *et al.* (2012), Sundaram *et al.* (2011), in fenugreek and by Zhong *et al.* (2021) in Capsicum frutescent, Kumar *et*

al. (2019) in *Allium sativum* L.

In our study, we found that the selection of specific primers, namely Satt 119 (Plate2) Satt 246, Satt 518 and Satt 448, yielded distinct and unique DNA bands. These bands serve as genetic fingerprints, facilitating the precise identification of individual genotypes within the Fenugreek population. The presence of such distinct bands signifies the genetic diversity and variability present among the studied genotypes.

Conclusion

In essence, the combination of molecular analyses provided a comprehensive overview of the genetic diversity present in Fenugreek genotypes. This knowledge has substantial implications for crop improvement programs, guiding breeding efforts and conservation strategies also identified fenugreek germplasm lines with superior character could be used in hybridization programme. The insights obtained from this study offer a solid foundation for future research and practical applications aimed at harnessing genetic diversity to develop improved Fenugreek varieties. The results contribute to the broader understanding of plant genetic resources and their potential for sustainable agriculture and food security.

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