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Original Article

ASSESSMENT OF GENETIC DIVERSITY OF TRIGONELLA FOENUM-GRACEUM L. IN NORTHERN INDIA USING RAPD AND ISSR MARKERS

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ABSTRACT

Objective: The main aim of this study was to assess genetic diversity and phylogenetic relationships of different varieties of fenugreek (8 varieties and 6 populations) collected from northern India using RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeat markers).

Methods: DNA Samples were analyzed with 400 RAPD and 100 ISSR primers. For statistical analysis data were subjected to UPGMA (Un weighted pair-group method with arithmetic averages), Jaccard's similarity coefficient values were obtained using Similarity for Qualitative Data (SIMQUAL), to find out genetic relationship. This analysis generated dendrogram which was further compared using the Mantel matrix correspondence test (NTSyS-pc version 2.02i) and Principal coordinates analysis.

Results: Polymorphism frequency of 42.91% and 55.66% was found in different varieties of fenugreek using RAPD and ISSR markers respectively. Based on the combined data of RAPD+ISSR marker system the maximum similarity index was observed for accessions from Mathura and RMT-143 (0.85) whereas the minimum similarity index was observed for RMT-351 sand RMT-303 (0.26).

Conclusion: The investigation has demonstrated that cluster analysis could be profitably used in unravelling the genetic variation within the accessions and the two molecular markers, RAPD and ISSR could be used as effective tools to evaluate genetic diversity and assess genetic relationship in fenugreek.

Keywords: Genetic diversity, ISSR, Polymorphism, RAPD, *Trigonella foenum-graecum* L.

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INTRODUCTION

The genus *Trigonella* is one of the largest genera of the tribe Trifoliate in the family Fabaceae and subfamily Papilionoideae [1]. Fenugreek (*Trigonella foenum-graecum* L. fam: Fabaceae) is an annual forage legume crop. The name *Trigonella* means "triangle shaped pale yellow flower" and *"foenum-graecum"* means "Greek hay" indicating its use as a forage crop [2]. It is supposed to be native to the Mediterranean region but now is grown as a spice in most parts of the world including Rajasthan, Gujarat, Uttaranchal, Uttar Pradesh, Madhya Pradesh, Maharashtra, Haryana and Punjab in India [3]. It is diploid and adapted to self-pollination [4] and fairly tolerant to frost and low temperature.

The major bioactive compound of fenugreek seeds are trigonelline and sapogenins. In china, the fenugreek seeds are used as a pessary to treat cervical cancer. In the Middle East and the Balkans, the aerial part of the plant is used for abdominal cramps associated with both menstrual pain and diarrhea or gastroenteritis [5] Antitumor, antioxidant [6], cholesterol-lowering activity, antidiabetic, antiplasmodial activity [7], anti hepatotoxic and nephrotoxic, anti-inflammatory [8], prophylactic activity [9] of fenugreek have reported by researchers all over the world. The mucilaginous seeds are used as a tonic, emollient, carminative, demulcent, astringent, expectorant, restorative, aphrodisiac and vermifugal properties, to cure mouth ulcers, chapped lips and stomach irritation [10].

According to Spice Board of India, annual production of fenugreek in India (2014), 110530 tons/90500 hectare land is expected, and it has a great overseas market demand. Basu *et al.*, 2007c [11] reported that fenugreek breeding was tried through various mutagens but the major problem comes intermediate growth habit and late maturity. The foundation for Innovation in medicine, USA has introduced a term recently, called nutraceuticals. Fenugreek finds use in the global nutraceutical industry, with India having the maximum share of these international markets.

Lack of information on genetic diversity and intra specific relatedness in *Trigonella* has limited scope for genetic improvement and hence effective conservation and management of its germ plasm resources. RAPD and ISSR markers have caught the fancy of many individuals in the field of applied plant breeding. RAPD was found to be simple and efficient among the available DNA-based techniques [12] and furthermore sequence information is not needed [13, 14]. With the availability of this genetic tool, genetic diversity and genetic analysis can also be estimated [15-18]. Furthermore, RAPD techniques are advantageous because of their simple requirement of a small quantity of DNA and their ability to uncover a large number of polymorphisms [19, 20].

The current investigation presents the results of the characterization of fourteen varieties of *T. foenum-graecum* collected from different locations of northern India using molecular analysis to understand their genetic richness and its diversity.

MATERIALS AND METHODS

Collection of plant material

A total of 14 varieties/populations [table 1] of *Trigonella foenum-graecum* L. (fam: Fabaceae) were collected from different locations in Northern India. Seeds of defined varieties grown in Rajasthan were procured from the Department of Plant Breeding and Genetics, S. K. N College of Agriculture, Jobner, Jaipur (Rajasthan) and Punjab Agricultural University, Ludhiana. Seeds of various populations were sampled from UP, MP, Punjab and Rajasthan and each accession was given a specific code.

PAU-Punjab agricultural university

DNA extraction

DNA was isolated from 5 g of leaf tissue grounded in liquid nitrogen. Total genomic DNA was extracted individually from younger leaves of different populations/varieties following the standard CTAB method [21].

PCR analysis (RAPD+ISSR)

PCR amplification was carried out according to the method of Williams [22] using 400 random decamer primers obtained from Operon Technologies (Alameda, USA). PCR amplifications were performed in a Gene Amp 9700 Thermal Cycler (Perkin Elmer Applied Biosystems) with an initial denaturation at 94 °C for 3 min followed by 45 cycles at 92 °C for 45 s, 37 °C for 30 s and 72 °C for 2 min with a final extension at 72 °C for 7 min.

For RAPD 5 ng of template DNA, 1x PCR buffer (10 mM Tris pH 9.0, 50 mMKCl, 1.5 mM MgCl2), 100 µM of each of the four dNTPs, 0.4 µM of RAPD primer and 0.3 Units of Taq DNA polymerase (Bangalore Genei, India). Similarly PCR amplification was performed in a 10 µl reaction mixture containing 5 ng of template DNA, 1x PCR buffer (10 mMTris pH 9.0, 50 mMKCl, 1.5 mM MgCl2), 0.2 µl of 25 mM MgCl2, 200 µM of each of the four dNTPs, 0.4 µM ISSR primer and 0.6 Units *Taq* DNA polymerase (Bangalore Genei, India) for ISSR.

The major difference between both reactions was the annealing temperature of ISSR reaction which was 37 ℃. The PCR amplified products obtained from RAPD and ISSR were electrophoretically separated in 1.7% agarose (Bangalore Genei, India) gels buffered with 1x TAE at 100 V for 3 h and visualized with ethidium bromide staining. The gel images were recorded using the Alpha Innotech Fluorchem gel documentation system.

Table 1: Varieties/Populations with their respective accession numbers and geographical coordinates

Code No.	Name of varieties/population	Latitude	Longitude
1	RMT-303 (Jobner)	26.970553	75.379110
2	Jaipur (Rajasthan)	26.912434	75.787271
3	RMT-1 (Jobner)	26.970553	75.379110
4	Sikar (Rajasthan)	27.609400	75.139911
5	RMT-143 (Jobner)	26.970553	75.379110
6	Mathura (Uttar Pradesh)	27.492413	77.673673
7	RMT-361 (Jobner)	26.970553	75.379110
8	M-150 (PAU)	30.902805	75.808641
9	T-8 (PAU)	30.902805	75.808641
10	RMT-305 (Jobner)	26.970553	75.379110
11	Kota (Rajasthan)	25.213816	75.864753
12	RMT-351(Jobner)	26.970553	75.379110
13	Suratgarh (Rajasthan)	29.320198	73.900709
14	Ratlam (Madhya Pradesh)	23.334170	75.037632

Statistical analysis

For each RAPD and ISSR primer, the presence or absence of bands in each accession was visually scored and set in a binary matrix. Similarity matrices were constructed using the Jaccard's similarity coefficient values using Similarity for Qualitative Data (SIMQUAL) [23] to find out genotypic relationship. The average distance of a single variety from rest of the genotypes was also calculated. The 0/1 matrix data obtained from RAPD primers was arranged to get separate similarity matrix which was subjected to UPGMA (un weighted pair-group method with arithmetic averages) analysis. This analysis generated dendrogram which was further compared using the Mantel matrix correspondence test (NTSyS-pc version 2.02i Principal coordinate analysis was performed, and the ordination displayed in two and three dimensions. In order to confirm the presence of bands and determine reproducibility, all the primers were replicated twice and if necessary thrice.

RESULTS

Assessment of genetic diversity in *T. foenum-graecum* L through RAPD markers

Out of the 400 tested primers, 28 primers gave very good polymorphism across all the accessions of fenugreek tested. The number of bands amplified per primer varied between 4 (OPM-7) and 16 (OPH-12) with an average of 9.32 bands per primer. A total of 261 bands were amplified of which 112 were polymorphic resulting in a polymorphism frequency of 42.91% and an average of 4 polymorphic bands per primer [fig. 1].

M=Molecular Weight Marker

1-RMT-303, 2-Jaipur, 3-RMT-1, 4-Sikar, 5-RMT-143, 6-Mathura, 7-RMT-361, 8-M-150, 9-T-8, 10-RMT-305, 11-Kota, 12-RMT-351, 13-Suratgrah, 14-Ratlam

The size of bands varied between 250 and 5000 bp. The similarity matrix was prepared based on Euclidian distances among different varieties and populations. The similarity coefficient value ranged between 0.19 to 0.87 %. The similarity was highest (0.87) between var RMT-143 and Mathura while it was least (0.19) between RMT-303 and RMT-351.

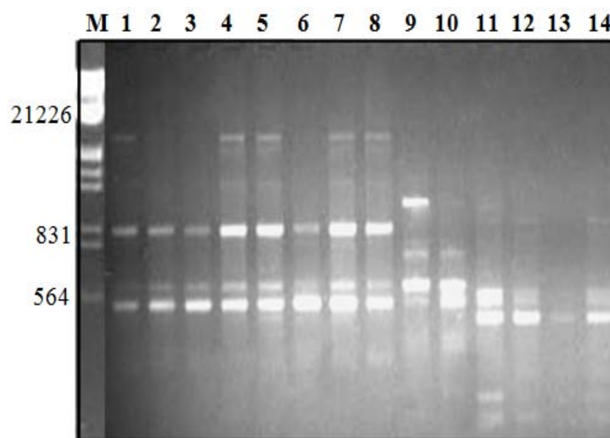

Fig. 1: RAPD analysis of 14 varieties/populations of *Trigonella foenum-graecum* L. using RAPD primers OPH-12

The cluster analyses revealed two groups (A and B). Group B is further divisible into two sub-clusters I, II. Sub-cluster I could be divided into two groups further (G1 and G2). Cluster A contained RMT-303; whereas rests of the varieties were included in cluster A. Subgroup, I had RMT-351. Sub-cluster II had RMT-1.

This sub-cluster was further divisible into II a, II b, II c and II d. The former two had MP and Suratgarh respectively, whereas II c con-

tained T-8, II d could be divided into II d 1 and II d 2 and II d 3 Whereas II d 1 had Kota and RMT-305, II d 2 had M-150 and RMT-361. Jaipur, Sikar, and RMT-143 and Mathura populations and one variety were placed in II d 3 [fig. 2].

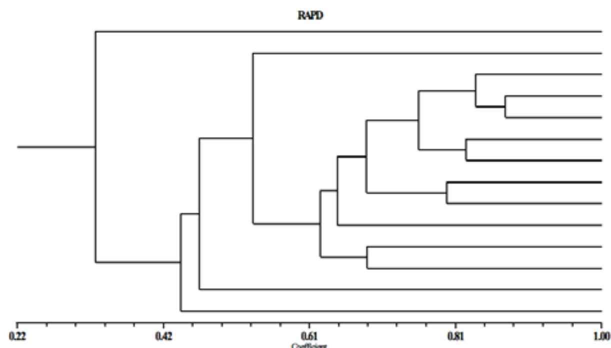

Fig. 2: Shows the clusters constructed through NTSYS (2.02 pc) drawn as dendrogram

ISSR analysis

Out of the 100 tested primers, 37 primers produced amplification products of which only 15 primers revealed polymorphic loci across the *T. foenum-graceum* tested. The number of bands amplified per primer varied between 2 (UBC-835) and 12 (UBC-862) with an average of 7.06 bands per primer. A total of 106 bands were amplified of which 59 were polymorphic resulting in a polymorphic frequency of 55.66 % and an average of 3.93 polymorphic bands per primer. The size of bands is varied between 200 and 3530 bp [fig. 3].

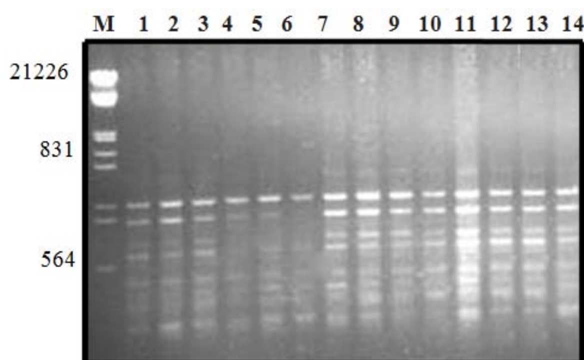

Fig. 3: ISSR analysis of 14 varieties/populations of *Trigonella foenum-graecum* L. using ISSR primer UBC-880

M=Molecular Weight Marker

1-RMT-303, 2-Jaipur, 3-RMT-1, 4-Sikar, 5-RMT-143, 6-Mathura, 7-RMT-361, 8-M-150, 9-T-8, 10-RMT-305, 11-Kota, 12-RMT-351, 13-Suratgrah, 14-Ratlam

The similarity matrix was prepared based on Jaccard's coefficient among different varieties and populations. The similarity coefficient value ranged between 0.30 to 0.81 %. The similarity was highest between var RMT-143 and Mathura while it was least (0.30) between RMT-1 and MP.

Fig. 4 shows the clusters constructed through NTSYS (2.02 pc) drawn as a dendrogram. The cluster analyses revealed two groups (A and B). Group B is further divisible into three sub-clusters I, II, III. Sub-cluster I could be divided into two groups further (1a and 1b). Cluster A contained MP population; whereas rest of the varieties were included in cluster B. Subgroup, I had RMT-305, RMT-351 and T-8. Sub-cluster II had RMT-303 and RMT-1. Subgroup III had Jaipur, Mathura and RMT-143 [fig. 4].

Combined RAPD and ISSR analysis

Based on the combined data of RAPD+ISSR marker system the maximum similarity index was observed for accessions from Mathura and RMT-143 (0.85) whereas the minimum similarity index was observed for RMT-351 sand RMT-303 (0.26).

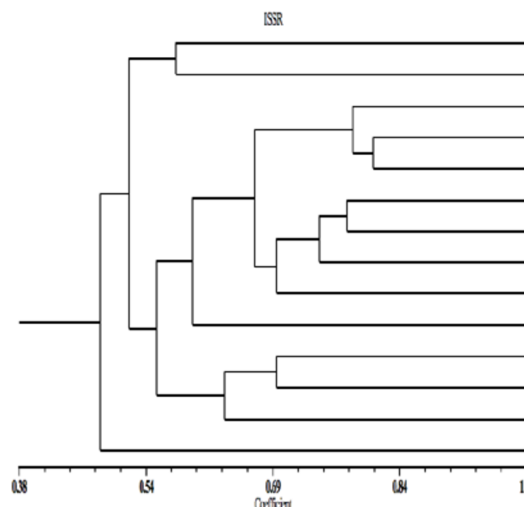

Fig. 4: Shows the clusters constructed through NTSYS (2.02 pc) drawn as dendrogram

The cluster analyses revealed two groups (A and B). Group B is further divisible into three sub clusters I, II, III. Sub cluster III could be divided into four groups further (1a, 1b, 1c and 1d). Cluster A contained RMT-303; whereas rests of the accessions were included in cluster B. Subgroup I had RMT-351. Sub cluster II had RMT-1. Subgroup III had four groups; 1a had MP and Suratgarh, 1b had RMT-305 and Kota and T-8, 1c RMT-351 and M-150, 1d had Jaipur, Sikar, RMT-143 and Mathura accessions [fig. 5].

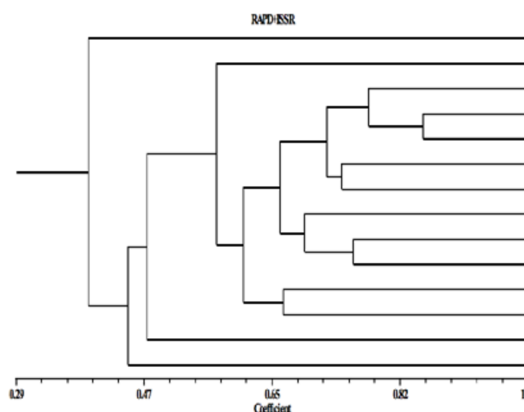

Fig. 5: Shows the clusters constructed through NTSYS (2.02 pc) drawn in the form of UPGMA dendrogram of 14 accessions collected from various locations

Fig. 6 and 7 shows PCO analysis based on RAPD+ISSR polymorphism grouping the various accessions in four clusters. Varieties RMT-303 could not be included in any specific cluster. Rest of the accessions could be grouped into three clusters; RMT-351 constituting a separate cluster.

Interestingly, in *T. foenum-graecum*, ISSR and RAPD could detect the almost comparable level of polymorphism. In the UPGMA analysis, *T.*

foenum-graecum with RAPD (42.91%) and with ISSR (55.66%) in accessions from Rajasthan and other places in Northern India. The adjacent region group e. g. Jaipur, Sikar, Suratgarh exhibited togetherness while they were placed in different clusters as compared with other instances e. g. MP, UP, etc. The similarity co-efficient values have suggested that *T. foenum-graecum* germ plasm collection represents a genetically diverse form attributed to high level of cross-pollination.

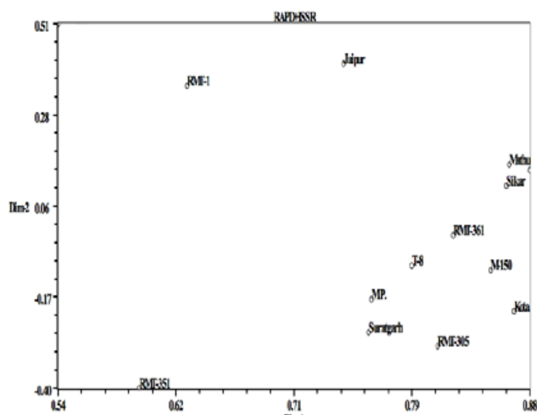

Fig. 6: Shows PCO analysis based on RAPD+ISSR polymorphism grouping the various accessions

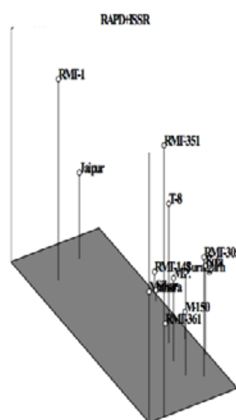

Fig. 7: Shows three-dimensional plot of 14 accessions and varieties by PCO analysis using Jaccard's similarity coefficient

DISCUSSION

RAPD and ISSR marker system based on PCR, due to its ease of use, reproducibility and requirement of the low amount of DNA got much popularity recently [24-27]. As is evident the number of accessions used by different workers varied, the largest number used was by [25]. Similarly, the number of RAPD primers used also varied. For instance, Dangi [24] investigated 100 whereas in the present study we employed 400 primers. Marzougui [28] have induced polyploidy in Tunisian populations of this species. Besides, the morphological features these workers analyzed cytological features in the induced polyploidy. Besides, the morphological features these workers analyzed cytological features in the induced polyploid. Similarly, [29] conducted assessment of relocation of *Trigonella cylindraceae L.* and *T. polyceratia* (L.) Trautv. *T. foenum-graecum* L. to Genus *Medicago* as inferred by RAPD and RFLP analyses. Dangi *et al.*[24]reported 70-72 % of polymorphisms using RAPD and ISSR marker system in collected germ plasm from different countries, on the other hand recently Tomar *et al.*[31] observed an average of 76.78% of polymorphism using RAPD in the 30 accessions collected from a western part of India. These contradictions in RAPD polymorphisms may be due to no uniform population and heterozygosity of fenugreek plants.

Recently, Naik [30] analyzed through ANOVA to measure the contributions of various variables to the observed association and partition the correlation coefficient into the components of direct and indirect effects. The intention of these workers was to estimate the degree and direction of association among various components of yield. Similar results were reported by other researchers also [24-26].

CONCLUSION

The similarity coefficient values have suggested that *T. foenum-graecum* germ plasm collection represents a genetically diverse form attributed to high level of cross-pollination. Molecular markers permitted to approximate the overall genetic diversity in *T. foenum-graecum* and concomitantly revealed molecular-based genetic relationship. The present study also demonstrated that *T. foenum-graecum* germ plasm in India constitutes a broad genetic base. From the clustering pattern and genetic relationship obtained with RAPD markers, breeders can fruitfully identify the diverse genotype from the different cluster and take them up in their future breeding program. The investigation has demonstrated that cluster analysis could be profitably used in unravelling the genetic variation within the accessions and suggested the probability of crop improvement through MAS and hybridization. The two molecular markers, RAPD and ISSR could be used as effective tools to evaluate genetic diversity and assess genetic relationship in fenugreek.

CONFLICT OF INTERESTS

Declared None

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