Diversity Evaluation of *Trigonella foenum-graecum* Populations Using DNA Markers and Phytochemical Characteristics

Daneshmand H (M.Sc. Student)[1], Etminan AR (Ph.D.)[1]*, Qaderi A (Ph.D.)[2]

1- Department of Biotechnology and Plant Breeding, Kermanshah Branch, Islamic Azad University, Kermanshah, Iran
2- Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran
* Corresponding author: Department of Biotechnology and Plant Breeding, College of Agriculture, Kermanshah Branch, Islamic Azad University, Kermanshah, Iran
Tel & Fax: +98-83-37243181
E-mail: alietminan55@yahoo.com

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Abstract

Background: Fenugreek (*Trigonella foenum-graecum* L.) is an important medicinal plant that is a source of diosgenin, trigonelline and other valuable components. Fenugreek seeds is used for its pharmaceutical and nutraceutical properties.

Objective: The aim of present study was to investigate the genetic diversity within and between different populations of *Trigonella foenum-graecum* using DNA markers and phytochemical characteristics.

Methods: The total genomic DNA was isolated from 12 populations of *Trigonella foenum-graecum* collected from different regions in Iran. After optimizing PCR, 10 ISSR and 10 SCoT primers were used to amplify the genomic DNA of all genotypes. To investigate genetic distance, cluster analysis based on Jaccard similarity coefficients and UPGMA algorithm calculated for the 12 genotypes. Also, the trigonelline content was determined between 2 populations which had the highest genetic distance.

Results: Analysis of banding patterns of 10 SCoT primers revealed 92 polymorphic bands with an average of 9.2 fragments per prime. A total of 79 bands were generated from 10 ISSR primers, out of which 55 (68.21%) were polymorphic bands. The average values of dissimilarities based on the SCoT and ISSR data were 0.60 and 0.44, respectively. Also, shoot trigonelline content was significantly different between genotypes with the highest genetic distance.

Conclusion: Our results revealed a high level of genetic variation among fenugreek populations. The findings also indicated that SCoT technique is more informative than ISSR technique for evaluation of genetic diversity and relationships among fenugreek populations. The genotypes with the highest genetic distance based on SCoT data, were noticeably different in respect of trigonelline content.

Keywords: ***Trigonella foenum-graecum* L., Genetic variation, ISSR, SCoT**

Introduction

Fenugreek (*Trigonella foenum-graecum* L.) belongs to the Fabacecae family which has been cultivated in Asia and Africa [1]. At present time, this plant is cultivated in large scale in Iran. Somatic chromosome numbers of Fenugreek was determined as 2n= 16 [2]. In Iran, Fenugreek exist wild in the provinces of Esfahan, Ardebil, Lourestan, Fars, Kerman, Baluchistan, Khorashan, Semnan, West and Eest Azarbaijan [3]. Fenugreek is source of diosgenin (a valuable pharmaceutical raw material) and other constituents such as trigonelline, protein, fixed oils, mucilage, as well as culinary uses for traditional and modern flavoring [4]. In Iranian traditional medicine the seeds are used as tonic and blood sugar lowering [5]. This herb has been used widely for therapeutic purposes. Fenugreek seed is recognized to show anti-diabetic, anti-nociceptive properties, hypocholesterolaemic, anti-cancer, and etc. [1].

Survival of a species depends on the management of germplasm and maintenance of genetic diversity within and among different populations. Genetic diversity is important because of its critical role in the survival and adaptability of a species. The evaluation of the genetic diversity is one of the main tasks of breeding programs because it may help in selection of cultivars and lines with higher diversity and better performance under specific conditions. Genetic variation might be evaluated by many different methods. Molecular markers are excellent tools to assess the amount of genetic variation [6]. DNA finger printing methods provide reliable means to study the genetic diversity and genetic relationships between different plant species. DNA markers play an important role in identification of superior genotypes and preservation of valuable germplasms.

Different types of DNA markers such as randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) have been developed to study the genetic diversity and relationships between plant populations. Inter simple sequence repeats (ISSR) constitute a simple and informative molecular marker technique for the rapid assessment of genetic diversity in plant species. The low reproducibility of RAPD, technical difficulties of AFLP and high development costs of SSR are the major limitations of these methods. ISSR marker is a technique that overcomes most of these limitations. Moreover, it is a very simple, fast, cost-effective, highly polymorphic and reliable method [7]. ISSR marker is detected by means of repeaters anchored primers that are amplified between SSRs. This technique exploits the abundant and random distribution of SSRs in plant genomes by amplifying DNA sequences between closely linked SSRs [8]. Furthermore this technique due to high repeatability and polymorphism as well as highly informative is suitable for assessing genetic diversity in different plants [9, 10]. In recent years, many new alternative and capable markers techniques have developed. Start Codon Targeted (SCoT) polymorphisms are reproducible markers that are based on the short conserved region in plant genes surrounding the ATG translation start (or initiation) codon [11]. SCoT markers have

been successfully used to evaluate genetic diversity and structure in different species, including wheat, rice, check pea, sugarcane and grape [11 - 16].

It has been reported that the kind and concentration of secondary metabolites varied among plant species. It is also reported that the may vary within the different parts of the plant. The genetic drift, physiological conditions, season, harvesting time and analytical method are effective factors on quality and quantity of secondary metabolites [17]. Many higher plants contain chemicals such as alkaloids, phenolics, terpenoids, flavonoids, coumarines, tannins, steroids and quinines that have allelopathic activity [18- 21]. Medicinal plants have also active substances with strong allelopathic properties [22, 23] that could be used safely in agro-ecosystems [24].

The aim of present study was to investigate: (1) the genetic diversity within and between different populations of *Trigonella foenum-graecum* using DNA markers, (2) comparison of SCoT and ISSR markers for measuring diversity and detection of genetic relationships and (3) determination of phytochemical characteristics between genotypes with the highest genetic distance.

Materials and Methods

Plant material and DNA isolation

The seeds of 12 populations of *Trigonella foenum-graecum* were obtained from Institute of Medicinal Plants - ACECR, Iran, which collected from different regions of Iran (Table 1). The seeds were sown under controlled conditions in nurseries to produce seedling plants. The total genomic DNA was isolated from young leaves of greenhouse-grown plants according to the CTAB protocol [25]. The quality of extracted DNA was tested on 0.8% agarose gel electrophoresis. The DNA samples were diluted and stored at -20 °C.

Table 1- Codes and localities of accessions.

Accession code	Locality	province	longitude	latitude	MASL*
P1	Ardestan	Isfahan	52° 23′	33° 23′	1250
P2	Isfahan	Isfahan	51° 40′	32° 37′	1550
P3	Ahvaz	Khouzestan	48° 40′	31° 20′	20
P4	Yazd	Yazd	54° 17′	31° 55′	1240
P5	Kerman	Kerman	56° 58′	30° 15′	1750
P6	Khash	Sistan va Balouchestan	61° 12′	28° 13′	1390
P7	Mashhad	Khorasan	59° 39′	36° 33′	1023
P8	Kermanshah	Kermanshah	47° 90′	34° 31′	1320
P9	Neyshabour	Khorasan	45° 48′	36° 16′	1210
P10	Zanjan	Zanjan	48° 29′	36° 41′	1660
P11	Semnan	Semnan	53° 32′	35° 35′	1130
P12	Yasouj	Kohgilouye va boyerahmad	51° 41′	30° 50′	1830

* Meter above Sea Level

ISSR-PCR

After optimizing PCR, a set of 10 ISSR primers (Table 2), was used to amplify the genomic DNA of all 12genotypes. The PCRs were performed in the reaction mixture 20 µl volume, with 2 µl of isolated DNA from each sample, 1.2 µl of each primer, 0.4 µl dNTPs Mix, 1.5 µl $Mgcl_2$, 5 units Taq polymerase (0.3 µl), 2 µl PCR buffer, and 12.6 µl ddH_2O (double distilled water). Amplification was run at 94 ºC for 4 min, followed by 30 cycles of denaturation at 94ºC for 30 seconds, primer annealing at 45-52ºC (considering Tm of primers) for 45 seconds and primer elongation at 72ºC for 2 min. The final extension was 7 min at 72ºC. The amplification reaction products were detected by 1.5% denaturing agarose gels stained with safe view II.

SCoT-PCR

A total of 10 SCoT primers were randomly selected (Table 2). The PCRs were performed in the reaction mixture 20 µl volume, with 10 µl master mix 2XPCR (Reddy to use PCR

Table 2- ISSR and SCoT primers and their amplification results generated in the 43 durum wheat germplasm

Marker	Primer	Sequence (5′→3′)	TAB	NPB	PPB	PIC	MI
ISSR	ISSR4	AGAGAGAGAGAGAGAGY	16	13	81.2	0.32	4.16
	ISSR5	ACACACACACACACACC	3	2	66.7	0.22	0.44
	ISSR7	CTCTCTCTCTCTCTCTG	6	6	100	0.39	2.34
	ISSR9	CACACACACACACACAG	8	5	62.5	0.29	1.45
	ISSR10	AGAGAGAGAGAGAGAGC	8	8	100	0.18	1.44
	ISSR11	ACACACACACACACACYA	7	4	57.1	0.32	1.28
	ISSR13	GAGAGAGAGAGAGAGAY	8	3	37.5	0.37	1.11
	ISSR15	ACACACACACACACACYG	7	4	57.1	0.33	1.65
	ISSR23	CTCTCTCTCTCTCTCTRC	6	3	50	0.38	1.14
	ISSR25	CACACACACACACACAR	10	7	70	0.31	2.17
	Mean		7.9	5.5	68.21	0.31	1.71
SCoT	SCoT2	CAACAATGGCTACCACCC	9	5	55.5	0.29	1.45
	SCoT3	CAACAATGGCTACCACCG	11	9	81.8	0.35	3.15
	SCoT4	CAACAATGGCTACCACCT	9	6	66.7	0.23	1.38
	SCoT5	CAACAATGGCTACCACGA	14	12	85.7	0.32	3.84
	SCoT6	CAACAATGGCTACCACGC	14	13	92.8	0.34	4.42
	SCoT7	CAACAATGGCTACCACGG	6	3	50	0.34	1.02
	SCoT8	CAACAATGGCTACCACGT	14	13	92.8	0.38	4.94
	SCoT9	CAACAATGGCTACCAGCA	11	10	90.9	0.32	3.2
	SCoT11	AAGCAATGGCTACCACCA	14	14	100	0.30	4.2
	SCoT25	CAATGGCTACCACTACAG	7	7	100	0.41	2.87
	Mean		10.9	9.2	81.63	0.32	3.04

Note: *TAB* total amplified bands, *NPB* number of polymorphic bands, *PPB* percentage of polymorphic bands, *PIC* polymorphism information content, *MI* marker index,
Note: Y= (C, T) and R= (A, G)

master mix 2X), 6 µl ddH_2O, 2 µl of isolated DNA from each sample and 2 µl of each primer. Amplification was run at 94 ºC for 5 min, followed by 45 cycles of denaturation at 94 ºC for 45 seconds, primer annealing at 45 ºC for 45 seconds and primer elongation at 72 ºC for 90 seconds. The final extension was 10 min at 72 ºC. The amplification reaction products were detected by 1.5% denaturing agarose gels stained with safe view II.

Trigonelline assay

For measurement of trigonelline in the shoot, the method of Zheng and Ashihara (2004) was modified the samples were ground with 80% methanol and magnesium oxide (MgO) in a mortar and pestle. After incubation at 60°C for 30 min, the homogenates were centrifuged and the supernatant was collected. After complete evaporation of methanol, the methanol-soluble extracts were dissolved in distilled water. The samples were filtered using a disposable syringe filter unit and the aliquots were used for determination of trigonelline (TG) by HPLC. The analyses of the samples were carried out using a Knauer K2600A liquid chromatography (Germany), equipped with a Nucleosil C18 (150 mm × 4.6 mm I.D, 5 µm) column. A mixture of methanol: water (50:50 v/v) served as the mobile phase and pH of solution adjusted to 5.0 with 50 mM sodium acetate [26].

Data analysis

Banding pattern of the SCoT (Figure 1) and ISSR (Figure 2) markers in the individuals were scored as presence (1) and absence (0) of the band. To investigate genetic distance, cluster analysis based on Jaccard similarity coefficients and UPGMA algorithm calculated for the 12 genotypes.

In order to measure the informativeness of the markers to differentiate between genotypes, polymorphism information content (PIC), marker index (MI) andresolvingpower (Rp) were calculated. PIC was calculated according to the formula of Anderson et al. [27], as $PIC = 1 - \sum pi^2$, where pi is the frequency of the ith allele of the locus in the 43 genotypes. MI was determined according to Varshney et al. [28]. The percent of shoot trigonelline between genotype-6 and genotype-8, which had the most genetic distance based on the molecular data, was compared by independent t-test.

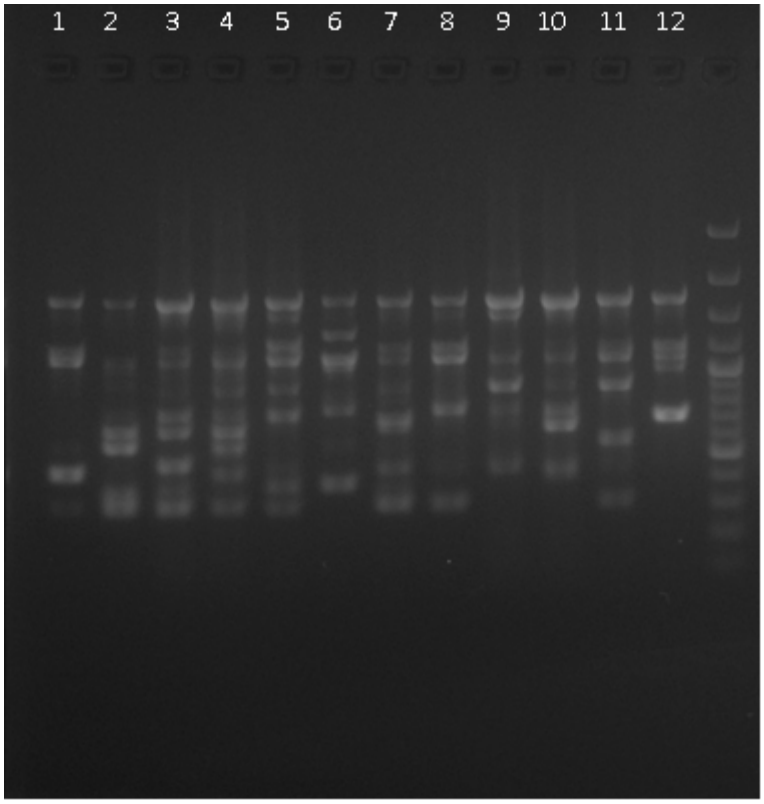

Figure 1- Amplified PCR products generated by primer SCoT-8 for 12 Fenugreek populations

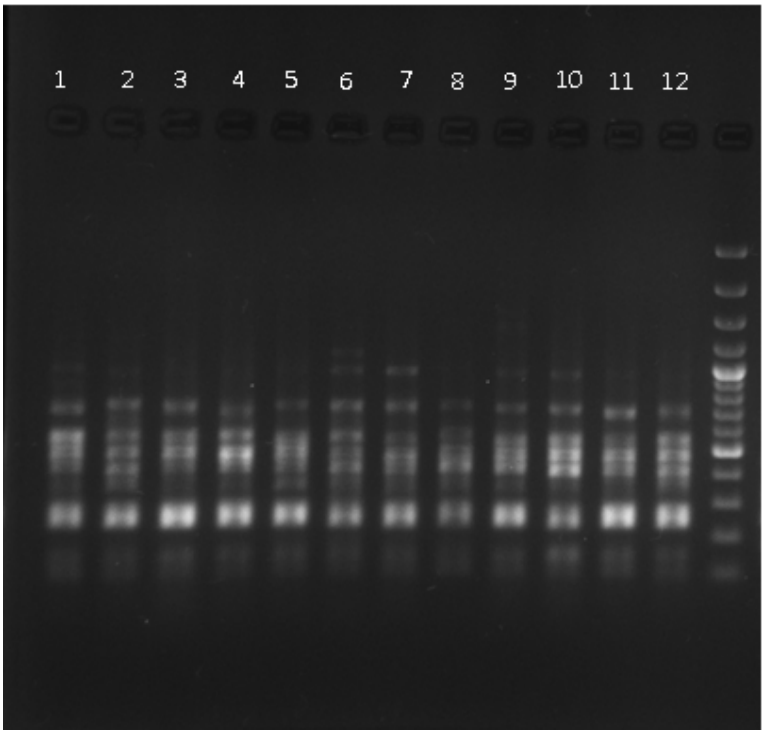

Figure 2- Amplified PCR products generated by primer ISSR-13 for 12 Fenugreek populations

Results

A total of 79 bands were generated from 10 ISSR primers, out of which 55 (68.21%) were polymorphic bands (Table 2). The number of polymorphic bands ranged from 2 (ISSR-5) to 13 (ISSR-4) with a mean of 5.5. The polymorphism information content (PIC) was used to characterize the efficiency of each primer to reveal polymorphic loci.PIC value ranged from 0.18(ISSR-10) to 0.39 (ISSR-7). The average of polymorphism information content index was 0.31 which is comparable to that found by Zamanifard et al. [29] using ISSR. The highest value (4.16) of marker index (MI) was observed for ISSR-4, while the lowest value (0.44) related to ISSR-5.

Ten SCoT primers were screened to investigate the genetic variation. The total bands per primer ranged from six (SCoT-7) to 14 (SCoT-11, 8, 6 and 5). Analysis of banding patterns of 10 SCoT primers revealed 92 polymorphic bands with an average of 9.2 fragments per prime. The highest and the lowest number of polymorphic bands per assay were 14 (SCoT-11) and 3 (SCoT-7) bands, respectively (Table 2).

The PIC values of the 10 primers varied from 0.23 (SCoT-4) to 0.41 (SCoT-25) with an average of 0.32. The highest and lowest value of MI was observed for SCoT-8 (4.94) and SCoT-7 (1.02) respectively.

To investigate genetic relationships among genotypes, cluster analysis based on Jaccard's similarity coefficients and UPGMA algorithm calculated for the 12 genotypes. According to the clustering pattern obtained by ISSR data, the 12 genotypes classified into four groups. According to the analysis, the first cluster consists of 7populations (Esfahan, Kerman, Khash, Kermanshah, Neyshabour, Semnan and Yasouj). Three populations (Ahvaz, Yazd and Zanjan) classified in the second group. Two other populations (Ardestan and Khorasan) were represented as individual branches in the cluster (Figure 3).

According to SCoT dendrogram, 6 populations (Ahvaz, Yazd, Kerman, Khorasan, Neyshabour and Zanjan) classified in a separated group. Two populations (Semnan and Yasouj) were classified in the same group. Finally the other populations (Kermanshah, Khash, Ardestan and Esfahan classified individually in separated groups (Figure 4).

The genetic distance estimated between pairs of genotypes, calculated based on ISSR and SCoT data is reported in Table 3 and Table 4 respectively. ISSR data showed the highest range of genetic distance among 12 accessions (from 0.27 up to 0.69) with an average of 0.44. Minimum, maximum and average values of dissimilarities based on the SCoT data were found to be 0.44, 0.79 and 0.60, respectively. These results revealed that SCoT data gave higher average genetic distance than ISSR data. Based on the phytochemical analysis, there was a significant difference ($P \leq 1\%$) between genotype-6 and genotype-8 for shoot trigonelline content (Table 5), (Figure 5).

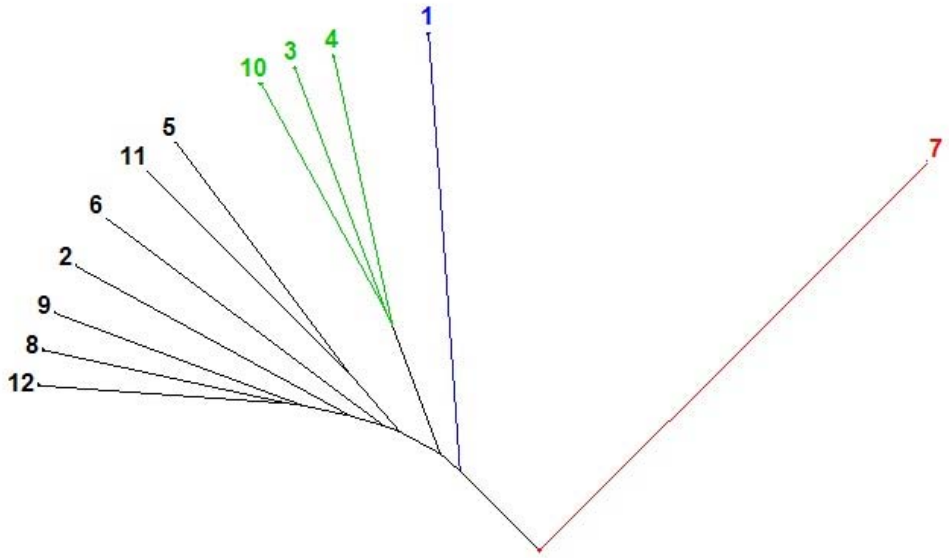

Figure 3- Dendrogram of 12 Fenugreek populations resulting from the UPGMA cluster analysis based on Jaccard's similarity coefficients obtained from ISSR marker

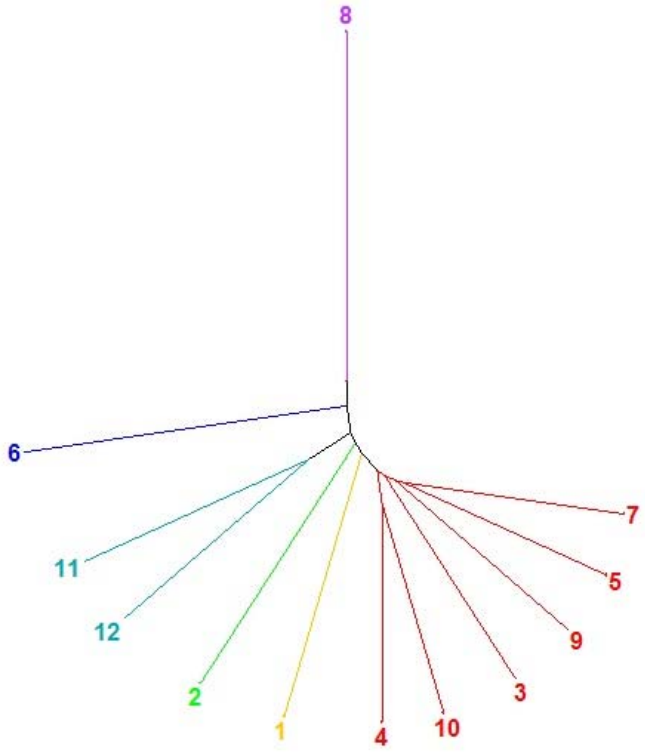

Figure 4- Dendrogram of 12 Fenugreek populations resulting from the UPGMA cluster analysis based on Jaccard's similarity coefficients obtained from SCoT marker

Table 3- Genetic distance matrix based on ISSR markers by Jaccard coefficient among 12 Fenugreek populations

	1	2	3	4	5	6	7	8	9	10	11	12
1	0											
2	0.49	0										
3	0.45	0.49	0									
4	0.50	0.50	0.31	0								
5	0.47	0.36	0.43	0.41	0							
6	0.52	0.41	0.55	0.53	0.36	0						
7	0.70	0.56	0.66	0.61	0.46	0.56	0					
8	0.56	0.36	0.49	0.54	0.39	0.37	0.64	0				
9	0.45	0.33	0.45	0.50	0.36	0.34	0.60	0.28	0			
10	0.46	0.42	0.28	0.29	0.34	0.46	0.52	0.45	0.36	0		
11	0.40	0.44	0.40	0.45	0.31	0.48	0.65	0.39	0.44	0.38	0	
12	0.51	0.33	0.41	0.49	0.28	0.37	0.55	0.27	0.29	0.38	0.36	0

Table 4- Genetic distance matrix based on SCoT markers by Jaccard coefficient among 12 Fenugreek populations

	1	2	3	4	5	6	7	8	9	10	11	12
1	0											
2	0.65	0										
3	0.55	0.57	0									
4	0.57	0.52	0.50	0								
5	0.59	0.61	0.48	0.51	0							
6	0.59	0.68	0.67	0.64	0.71	0						
7	0.59	0.59	0.48	0.56	0.46	0.60	0					
8	0.76	0.79	0.71	0.72	0.68	0.80	0.65	0				
9	0.53	0.58	0.54	0.51	0.49	0.65	0.46	0.67	0			
10	0.55	0.59	0.53	0.45	0.51	0.66	0.56	0.75	0.45	0		
11	0.70	0.66	0.54	0.63	0.61	0.78	0.56	0.70	0.60	0.66	0	
12	0.70	0.64	0.55	0.64	0.58	0.69	0.56	0.66	0.56	0.62	0.51	0

Table 5- Comparison the average of shoot trigonelline content between genotypes with the most genetic distance based on the SCoT data

Genotype	Shoot Trigonellin (%)	Std. Error Mean	t	Sig. (2-tailed)
Genotype -6	0.319	0.0029	10.19	0.001
Genotype -8	0.349	0.0006		

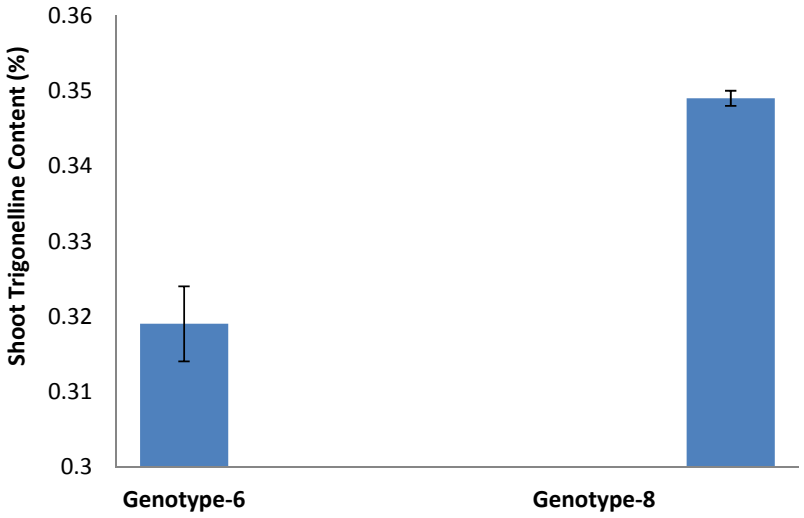

Figure 5- The production of Trigonelline in genotype-6 and genotype-8

Discussion

Information about the degree and distribution of genetic variation and relationships among breeding materials has a great value in crop improvement programs. ISSR and SCoT markers have been used successfully to determine genetic variation and relationships in many plants [12, 30, 31, 32, 33, 34, 35]. Some previous studies have reported that these molecular markers are useful tools for assessment of genetic diversity because of their high reproducibility and great power for the detection of polymorphism [14, 15, 36, 37]. In this study, the genetic diversity among some Iranian populations of fenugreek was investigated by SCoT in comparison with ISSR markers. ISSR markers are known to be abundant, very reproducible, highly polymorphic, highly informative and quick to use [9]. SCoT is one of the targeted molecular marker systems, which characterized by simplicity and reproducibility [14]. In the present study the efficiency of both ISSR and SCoT markers estimated through parameters such as polymorphism information content (PIC) and marker index (MI). These parameters have been used usually for evaluating the informative potential of DNA markers in different germplasm and cultivated genotypes. [38, 39, 40, 41]. The moderate value of PIC was estimated for both markers showing the efficiency of the used primers in discrimination of the individuals. The MI, which can be considered to be a common measure of efficiency in discovering polymorphism [42], was different in two marker systems (Table 2). Based on the results, the marker index of SCoT primers was higher than ISSR primers (Table 2). MI can be proposed as most marker parameters for selecting informative primers. Thus primer SCoT-6 and SCoT-8 were identified as the best primers for assessment of genetic diversity in Fenugreek germplasm. On the other hand, SCoT as a gene-targeted marker is more desirable for various applications in plant

molecular genetics especially, QTL mapping since recombination levels between gene and marker or QTL are lower compared to RAPDs, SSRs or ISSRs [43]. SCoT marker system is a novel technique and detects polymorphisms in the short conserved region in plant genes surrounding the translation start codon. Compared with anonymous markers, SCoT may yield more accurate estimates of genetic variability [11]. Phytochemical analysis of two selected genotypes revealed that the SCoT marker system could recognize the phenotypical variation, however, for certain viewpoint more researches are required.

Conclusion

In conclusion, we have shown that ISSR and SCoT markers can be used for diversity analysis in fenugreek populations. The results revealed a high level of genetic variability among fenugreek populations based on two genetic marker systems. The efficiency of SCoT markers for analyzing the genetic diversity and relationships among fenugreek populations was another remarkable result obtained from this study. Our results confirm the relevance and suggest the effectiveness of the SCoT markers for assessing genetic diversity in fenugreek populations. Trigonelline content was significantly different ($P \leq 1\%$) between genotype with the heist genetic distance. Regarding the high genetic distance estimated between two populations of fenugreek which were significantly different for trigonelline content, it is concluded that phytochemical characteristics such as trigonelline content is controlled genetically. These results revealed that the selection of fenugreek populations for higher trigonelline content requires accurate estimates of genetic diversity and relationships. Base on genetic distance and phytochemical different between fenugreek population is expected to increase rates of genetic gain by selection programs.

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