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Full Length Article

Assessment of Genetic Diversity in Fenugreek (*Trigonella foenum-graecum*) in Oman

Issa Talib Al-Maamari[1], Abdullah Mohammed Al-Sadi[2*] and Nadiya Abubaker Al-Saady[3]
[1]*Masart Alandhar Farm, Royal Court Affairs, Suwaiq*
[2]*Department of Crop Sciences, College of Agricultural and Marine Sciences, Sultan Qaboos University, P.O. Box 34, AlKhoud 123, Oman*
[3]*The Animal and Plant Genetic Resources Center, The Research Council, Oman*
*For correspondence: alsadi@squ.edu.om

Abstract

Fenugreek (*Trigonella foenum-graecum* L.) is widely grown in the Arabian countries and is one of the important crops in the Sultanate of Oman. The present investigation focused on characterizing genetic diversity within 20 Omani fenugreek accessions collected from different districts in Oman and to investigate their relationship with four accessions from Iraq and Pakistan. AFLP analysis of 24 accessions produced 1852 polymorphic loci. The level of genetic diversity (H) was found to be 0.2146, 0.0844 and 0.1620 for the Omani, Pakistani and Iraqi populations, respectively. The moderate level of genetic diversity of fenugreek in Oman indicates that it has been cultivated in the country for long time. A very low level of genetic differentiation was observed among populations of fenugreek from different regions in Oman (Fst = 0.05) following AMOVA analysis. Cluster analysis supported these findings and indicated high genetic similarity among Omani populations of fenugreek (mean = 93%) compared to a lower level of genetic similarity with the population from Pakistan (83%) and Iraq (80%). These results suggest frequent exchange of fenugreek genetic material among regions in Oman.

Keywords: AFLP Fingerprinting; Accessions; Hilbeh; Hulbah

Introduction

Trigonella foenum-graecum L., commonly known as fenugreek and locally as "Hilbeh" or "Hulbah". It is planted in various countries (Kawashty *et al.*, 1998; Khan *et al.*, 2005; McCormick *et al.*, 2009). The origin of fenugreek is assumed to be in the Mediterranean region and adjacent areas (Duke *et al*., 1981). Fenugreek is widely cultivated in different parts of the world, including India, Egypt, Ethiopia, and England. It can enrich soil, fix nitrogen and used for animal field and human consumption (Bromfield *et al*., 2001). Fenugreek seeds supplies dietary proteins for vegetarians that lack animal and fish protein in their diet. Furthermore, fenugreek has several medicinal benefits (Sharma, 1990; Srinivasan, 2014).

Oman has a high level of diversity in terms of cultivated and wildly grown plant species. Fenugreek is one of the important plant species in Oman and is mostly dominated by indigenous unknown accessions (Al-Saady *et al*., 2012). It is commonly grown in five main regions in Oman: Dakiliya, Dhahira, Buraimi, Batinah and Sharqiya.

Little information is available concerning the origin of fenugreek in Oman. It is assumed that like some other crops in the country, fenugreek has been introduced from the Indian sub-continent since trade has been very active with this part of the world over the past 5 centuries (Al-Sadi *et al*., 2012b). Since no information is available concerning genetic diversity of fenugreek in Oman, it is not clear whether fenugreek accessions consist of one or several genotypes. In addition, little information is available concerning relationship of fenugreek from different parts of the country with each other and with exotic populations. This establishes a barrier towards future breeding and improvement programs of indigenous cultivars. In addition, the recent introduction of commercial fenugreek cultivars may replace indigenous cultivars that have adapted to the local conditions.

The use of molecular markers is common in genetic diversity studies within populations of several plant and microorganism species (Jinping *et al.*, 2009; EL-Mouei *et al.* 2011; Al-Sadi *et al.*, 2012a, 2012b, 2012c; Al-Sadi, 2013). Molecular markers can detect polymorphism within nucleic acid sequences and help in crop production improvement strategies and in breeding programs. Limited previous studies addressed genetic diversity within populations of fenugreek using ISSR, RAPD and AFLP techniques (Dangi *et al.*, 2004; Kakani *et al.*, 2011; Kumar *et al.*, 2012).

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This study was carried out to investigate genetic diversity of fenugreek accessions in Oman using AFLP fingerprinting. Specific objectives were to study genetic diversity of fenugreek and to characterize relationship among fenugreek populations from regions in Oman. Knowledge in these areas will help establish a basis for future breeding and conservation programs for fenugreek in Oman.

Materials and Methods

Collection of Samples

Twenty Omani accessions of fenugreek (*Trigonella foenum graecum* L.) were obtained from the Gene Bank of the Agricultural Research Center, Ministry of Agriculture and Fisheries, Oman (Table 1; Fig. 1). Fenugreek seeds were planted at the Masarat Al Andhar (MA), Royal Court Affairs site in Willayat Al Suwaiq (23º 48.28 longitude and 57º 17.33.4 latitude with an altitude of 80 m above mean sea level). In addition, 4 fenugreek accessions obtained from Iraq (2) and Pakistan (2) were included in the study. Three to five young leaves, randomly collected from each accession were used for molecular analysis.

Nucleic Acid Extraction

Extraction of DNA was done using CTAB method with some modifications (Doyle and Doyle, 1987). Fresh young leaves were randomly collected from fenugreek plants raised in a farm. The fenugreek leaves were kept in ice box and stored at -80°C until further used. Approximately three grams of three weeks old leaf tissues from each accession were ground using pre-chilled electrical drill machine with a glass-made nail with 200 µL extraction buffer (EB) containing 1% CTAB, 0.02 M EDTA, 1.4 M NaCl, 0.1 M Tris HCl pH 8.0, 0.1% v/v of ß-mercaptoethanol and 0.5% w/v PVP. Additional 300 µL of EB and 40 µL of SDS (10%) were added after grinding the leaves followed by incubation at 65°C for 60 min. Phenol: chloroform: isoamyl alcohol (25:24:1) was added and the supernatant was collected in new 1.5 eppenddorf tubes. Equal amount of chilled isopropanol and 0.3 M NaAc (pH 5.2) was added to each tube and placed at -20°C for 30 min. This was followed by centrifugation at 10000 rpm and 4°C for 20 min. The DNA pellets were washed twice using 70% ethanol and then dissolved in 50 µL TE buffer (10 mM Tris, 1 mM EDTA pH.8). Total amount of DNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

Amplified Fragment Length Polymorphism (AFLP)

The genetic diversity of fenugreek was assessed using AFLP (Vose *et al.*, 1995; Al-Sadi *et al*., 2012a). Genomic DNA (2 µL), obtained from 24 local and exotic accessions,

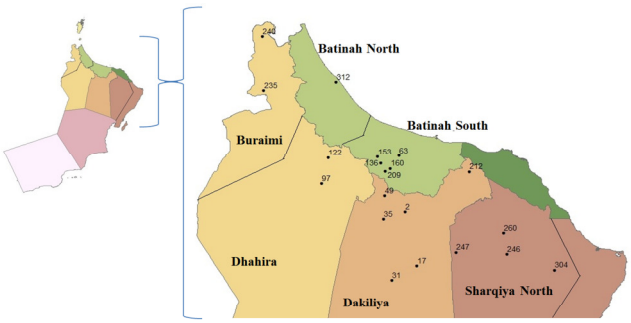

Fig. 1:A map of Oman showing the six governorates from which 20 fenugreek accessions were collected.

Table 1: Characteristics of *Trigonella foenum graecum* L. accessions used in the study

Accession No.	Region	Wilayat	Village
312	Batinah North	Sohar	Al-Ghudafa
63	Batinah South	Rustaq	Haat
136	Batinah South	Rustaq	WadiBaniAouf
153	Batinah South	Rustaq	WadiBaniGhafer
160	Batinah South	Rustaq	Aldhahir
209	Batinah South	Rustaq	WadibaniGhafer
235	Buraimi	Buraimi	Al-Hail
240	Buraimi	Muhadha	Al-Khabeen
122	Dhahira	Yanqul	Al-Bouwerdah
97	Dhahira	Ibri	Asubal
2	Dakiliya	Nizwa	Tanuf
17	Dakiliya	Manah	Al-Blaad
31	Dakiliya	Adam	Al-Belad
35	Dakiliya	Bahla	Al-Khatwa
49	Dakiliya	Al-Hamra	Al-Qlaah
212	Dakiliya	Bidbid	Al-Buwareed
246	Sharqiya North	Al-Qabel	Bateen
260	Sharqiya North	Ibra	Al-Haimah
274	Sharqiya North	Mudhaibi	WadiEndam
304	Sharqiya North	WadiBaniKhaled	Halfah
117722	Punjab	Pakistan	-
117702	Punjab	Pakistan	-
110364	Dahuk	Iraq	-
66620	Ninwa	Iraq	-

(-) unknown

was digested in mix containing 2.5 µL of 10X reaction buffer, 1 µL *Eco* R1 (10 µM) and *Mse*I (10 µM) enzyme combination mixture and sterile distilled water (SDW) up to a volume of 11.5 µL. The samples were incubated for 3 h at 37°C and then for 15 min at 70°C to inactivate the enzyme. AFLP ligation was carried out with 12 µL of adaptor/ligation solution and with 0.5 µL T4 DNA ligase (Fermentas, USA). The DNA digestion-ligation product was diluted 1:5 with TE buffer.

AFLP Pre-Amp Primer Mix I (Invitrogen, USA) was used following the manufacturer's protocol. The reaction mixture consisted of 2.5 µL of diluted adapter-ligated DNA and 22.5 µL of AFLP pre-amplification mixture containing primers carrying one selective nucleotide each [(1 µL *Eco RI* + A (10 µM), 1 µL *Mse*I + C (10 µM)], 2.5 µL PCR reaction buffer 10X, 0.25 µL Taq DNA

polymerase (5 U μL^{-1}), 1 µL dNTPs (5 mM), 2 µL MgCl (25 mM) and ddH_2O up to a volume of 25 µL. Polymerase chain reaction conditions were as follows:94°C for 2 min, followed by 20 cycles at 94°C for 30 sec, 56°C for 1 min and 72°C for 1 min and a final extension at 72°C for 7 min. The products were diluted 1:20 with TE buffer.

Analysis of genetic diversity within fenugreek accessions was done using six primer-pair combinations (Table 2). The selective PCR mixture contained 7µL of diluted pre-selective reaction mixture, 1 µL of *Mse*I+3 primer (10 µM), 1 µL *Eco* RI+3 primer (10 µM) (with three selective nucleotides), 1 µL dNTPs (5mM), 2 µL $MgCl_2$ (25 mM), 0.25 µL Taq DNA polymerase (5 U μL^{-1}), 2.5 µL PCR reaction buffer 10X and ddH_2O up to a volume of 25 µL. Denaturation in PCR was at 94°C for 2 min which was followed by 94°C for 30 sec, annealing temperature in the first cycle of 65°C for 30 sec (lowered by 0.7°C per cycle) and elongation of 10 min at 72°C for the next 12 cycles. This was followed by 23 cycles at 94°C for 30 sec, 56°C for 30 sec and 72°C for 2 min followed by extension for 10 min at 72°C. The final product was stored at -20C° till further processed.

Fragment and Data Analysis

A total of 0.15 μL Liz (Gene Scan™ - 500 LIZ™, Applied Biosystems™, UK) and 10 μl Formamide (Hi-Di™ Formamide, Applied Biosystems™, UK) were added to each 1.5 μL DNA of each accession in a plate. The mixture was placed after denaturation in a 3130 Genetic Analyser (Applied Biosystems™, Hitachi-Japan).

AFLP data within the range 50 - 500 bp was analyzed by the Gene Mapper 4.0 software. The number and percentage of polymorphic loci and Nei (1973) gene diversity were determined for each population (Al-Sadi *et al*., 2012a). UPGMA dendrogram was constructed (Nei, 1978) and variation among and within populations of fenugreek was determined using analysis of molecular variance (AMOVA; Arlequinv. 3.1) (Excoffier *et al*., 2005).

Results

AFLP Primer Pair Combinations

AFLP analysis of 24 fenugreek accessions using 6 primers resulted in 1852 alleles. The number of polymorphic loci ranged from 126 to 403 for the different primers (Table 2). Polymorphic loci ranged from 99.1% to 100% and Nei (1973) gene diversity ranged from 0.1448 to 0.2481. The primes E+AAC/M+CTT (0.2481), E+AAC/M+CAC (0.2475) and E+AAG/M+CAC (0.2458) gave the highest Nei gene diversity estimates (Table 2).

Genotypic and Genetic Diversity

Analysis of the 24 fenugreek accessions showed that they consist of 24 genotypes. The overall level of Nei's gene diversity was 0.2146 for the population from Oman (Table 3). Gene diversity estimates ranged from 0.0784 for Dhahira population to 0.1857 for Dakiliya population.

Table 2: Genetic diversity estimates in the population of *Trigonella foenum-graecum* using 6 primer pair combinations

No	*Ec*RI	*Mse*I	NPA	PPA	H
1	AAC	CAG	286	100	0.2266
2	AAC	CTT	344	99.1	0.2481
3	AAC	CTC	342	99.1	0.2303
4	AAC	CAA	126	100	0.1448
5	AAC	CAC	403	100	0.2475
6	AAG	CAC	351	99.2	0.2458

NPL is the number of polymorphic loci, PPLis the percentage of polymorphic loci and HisNei (1973) gene diversity

Table 3: Population genetic analysis of *Trigonella foenum-graecum* L. from Oman, Pakistan and India

Population	Sub-population	N	g	NPL	PPL	H
Oman	Oman	20	20	1544	83.4	0.2146
	Batinah South	5	5	1032	55.7	0.1826
	Buraimi	2	2	450	24.3	0.1008
	Dhahira		2	349	18.8	0.0784
	Dakiliya	6	6	1105	59.7	0.1857
	Sharqiya North	4	4	782	42.2	0.1531
Pakistan	-	2	2	376	20.3	0.0844
Iraq	-	2	2	721	38.9	0.1620
Overall	-	24	24	1852	100	0.2351

Where N is the sample size; g is the number of genotypes; NPL is the number of polymorphic loci, PPL is the percentage of polymorphic loci (out of 1852); and H is Nei(1973) gene diversity.

Table 4 Variation as measured using AFLPs among and within populations of Omani *Trigonella foenum graecum* L. and accessions from Pakistan and Iraq based on hierarchical analysis of molecular variance (AMOVA)

Source of Variation	f.	Sum of square	Variance component	Percent variation	st	P value	Gene flow
Oman							
Among	4	1112.767	12.43933	5.11	0.0511	0.0039	.64
Within	15	3463.833	230.92222	94.89			
Total	19	4576.60	243.4289				
Countries							
Among		1134.775	92.3816	27.46	.2746	<0.0001	0.66
Within	21	5125.100	244.0538	72.54			
Total	23	259.875	336.43384				
All							
Among	6	2226.139	42.0722	15.02	.1502	<0.0001	1.1
Within	16	3809.600	238.10000	84.98			
Total	2	6035.739	80.17202				

Genetic Similarity

Omani populations of fenugreek shared 88 to 96% (mean 92%) genetic similarity among each other. They shared 80-

Table 5: Pairwise genetic differentiation among populations of Omani *Trigonella foenum graecum* L. and accessions from Pakistan and Iraq

Accessions	Batinah-S	Buraimi	Dhahira	Dhakiliya	Sharqiya-N	Pakistan	Iraq
Batinah-S	------	18.59	4.09	6.74	4.35	0.87	0.71
Buraimi	-0.01363	---------	2.15	15.66	3.32	0.64	0.70
Dhahira	0.05754	0.10426	-------	10.96	1.27	0.43	0.54
Dhakiliya	0.03575	0.01571	-0.02334	-------	3.03	1.04	0.61
Sharqiya-N	0.05433	0.06994	0.16415	0.07620	-------	0.58	0.46
Pakistan	0.22341	0.27986	0.36515	0.19453	0.29974	--------	0.57
Iraq	0.25920	0.26352	0.31498	0.29206	0.35066	0.30614	------

Below diagonal: F_{ST} value, Above diagonal: gene flow (Nm) values

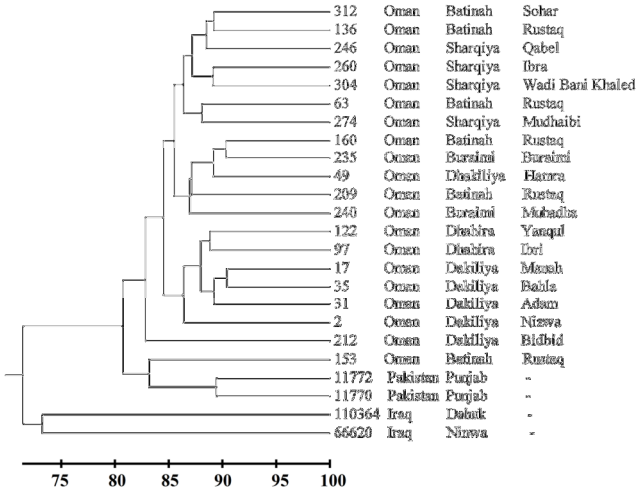

Fig. 2: UPGMA dendrogram illustrating Nei (1978) genetic similarity of 24 fenugreek accessions from different regions in Oman and from Pakistan and Iraq.

Table 6: Pairwise genetic differentiation among populations of *Trigonella foenum-graecum* L. and accessions from Pakistan and Iraq

Region	Oman	Pakistan	Iraq
Oman	-------	0.93	0.53
Pakistan	0.21149	--------	0.57
Iraq	0.32216	0.30614	----------

Below diagonal: F_{ST} value, Above diagonal: gene flow (Nm) values

85 (mean 83%) genetic similarity with the Pakistani population and 75-86 (mean 80%) genetic similarity with the Iraqi population (Fig. 2).

Cluster analysis showed that Omani accessions of fenugreek clustered together and were separated from Iraqi accessions. Pakistan accessions formed a separate cluster but they were closely related to one accession from Batinah, Oman (Fig. 2). Most accessions obtained from Dakiliya and Dhahira clustered in one clade while most accessions from Batinah, Buraimi and Sharqiya clustered together (Fig. 2).

Partition of Genetic Variation

According to the results generated through analysis of molecular variance (AMOVA) of Omani fenugreek accessions, 5.1% of genetic variation was found among local populations obtained from different regions in the country. There was very low levels of genetic differentiations among all regions in Oman (-0.01363 to 0.10426) except for moderate levels of genetic differentiation between populations from Sharqiya North and Dhahira (0.16415). On the other hand, high levels of genetic differentiation (27.46%) were observed between the Omani and exotic populations of fenugreek (Tables 4, 5, 6). Pairwise genetic differentiation values among Omani, Iraqi and Pakistani populations were high, except for Batinah South and Dakiliya regions, which were found to have moderate levels with the Pakistani population (Table 5).

Discussion

The genetic resources of indigenous underutilized species face extinction due to several factors, which include changes in farming systems, food habits, climate change and the introduction of high yielding crops (Zahoor, 2007; Jiang *et al*., 2014). The lack of information and genetic studies on many of the landraces of these crops, as with the case of fenugreek in Oman, has established a barrier towards further improvements and selection in this important crop.

Findings from this study provided evidence for low to moderate levels in the genetic diversity of the Omani populations of fenugreek (H = 0.0784-1857; overall 0.2146). These levels were comparable to the levels which have been reported previously for 17 fenugreek accessions from different countries (0.211) (Dangi *et al.,* 2004), 5 accessions for India (0.238) (Kumar *et al*., 2012) and 17 accessions from India (0.162) (Kakani *et al*., 2011). These levels were also expected for an annual self-pollinated crop. The higher level of genetic diversity within populations of fenugreek from Batinah South and Dakiliya regions compared to other regions in Oman may indicate that fenugreek has a longer history of cultivation in these areas and could be the main center of diversity of fenugreek in Oman. This is consistent with the production of fenugreek in these areas, which are known to be the two main areas for production of fenugreek in Oman. Areas with higher level of genetic diversity are most likely to be the center of origin or diversity of a certain species (Ferreira *et al.*, 2010; Al-Sadi *et al.*, 2012a,b; Al-Sadi, 2013).

AMOVA analysis revealed that the genetic differentiation values are low among populations of fenugreek from Oman. These findings were supported by the high level of genetic similarity among these populations and by cluster analysis. These findings may indicate that fenugreek accessions in Oman originated from similar sources. The low genetic differentiation values between populations from Dakiliya and Dhahira, two adjacent regions, may indicate that there has been frequent exchange of fenugreek germplasm between the two regions. The exchange of agricultural material among regions in Oman is common and has been suggested to be responsible for spread of several diseases in the past (Al-Sadi *et al.*, 2012b; Al-Sadi, 2013).The high level of genetic differentiation between Omani populations and the populations from Iraq and Pakistan may indicate that these populations evolved independently. However, the small sample size of accessions from Iraq and Pakistan makes it difficult to predict the extent of the relationship between populations from these countries. Future studies might be required to investigate the extent of this relationship by including more samples from Iraq and Pakistan.

AFLP analysis of fenugreek using 6 primer pair combination produced 1852 polymorphic loci (100% polymorphism across all accessions and primers). The number and percentage of polymorphic loci (PL) is higher compared to values obtained by Kumar *et al*. (2012) (AFLP = 64%), Kakani *et al.* (2011) (RAPD = 65%) and Dangi *et al.* (2004) (ISSR + RAPD = 71%). This supports previous findings of high resolution of AFLP in analyzing genetic diversity levels (Pang *et al.*, 2007; Al-Saady *et al.*, 2010; Al-Sadi *et al.*, 2012b).

This is the first study to characterize genetic diversity among fenugreek populations in the Arabian Peninsula. Findings from this study establish that the genetic diversity is moderate within the population of fenugreek in Oman. It also provides evidence for a relationship between fenugreek populations from different regions in Oman. Due to the low sample size from Iraq and Pakistan, further studies might be required to characterize the extent of relationship among Omani, Pakistani and Iraqi populations. A study is in progress to characterize phenotypic diversity among fenugreek accessions in Oman.

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