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Comparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis and restricted fragment length polymorphism among fenugreek accessions

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ABSTRACT. Protein and DNA polymorphismswere surveyed among seven accessions of wild fenugreek (*Trigonellafoenum-graecum* L.) to estimate their genetic diversity and relationships. Samples were obtained from diverse ecogeographical areas in Saudi Arabia and Yemen. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of seed storage protein showed genetic variations among fenugreek germplasms, both quantitatively and qualitatively, generating a total of 168 polypeptide bands with different molecular weights ranging from 4.5 to 300 kDa. Twenty-six of these bands were polymorphic, with a considerable polymorphism value (80.00%). Furthermore, restriction fragment length polymorphism (RFLP) analysis was also employed, which was based on the ability of four restriction enzymes (*Eag*I, *Eco*RI, *Fsp*I, and *Hin*dIII) to cleave genomic DNA of the plant materials at specific target nucleotide sequences into different

numbers of DNA fragments. RFLP analysis revealed 166 fragments with known sequences and variable lengths ranging from 80 to 4000 bp with a highly degree of polymorphism (88.71%). Data derived from SDS-PAGE or RFLP analyses were used to produce dendrograms, which clustered the studied fenugreek accessions into different groups based on the unweighted pair group method with arithmetic mean (UPGMA). The resulting relationships indicated that these two marker techniques were nearly equivalent, but not identical, with respect to phylogenetic information. In conclusion, SDS-PAGE analysis of seed proteins should be augmented with RFLP analysis of DNA for reliable estimates of genetic diversity among fenugreek germplasms.

Key words: Fenugreek; Genetic diversity; Polymorphism; Cluster; SDS-PAGE; RFLP

INTRODUCTION

Fenugreek (*Trigonella foenum-graecum* L.) is an herbal and annual flowering medicinal legume crop that belongs to the family Leguminosa. Its seeds are used in many Asian countries as a spice in food preparations due to their strong flavor and aroma. Furthermore, it is considered to possess therapeutic and medicinal properties in many parts of the world (Khoja et al., 2011). Fenugreek is also used as an herbal medicine due to its carminative, tonic, and aphrodisiac effects (Xue et al., 2007). Fenugreek seeds exhibit hypoglycemic, hypolipidemic, antifertility, antiandrogenic, antinociceptive, and wound healing properties, and are a good source of dietary fiber (Abou El-Soud et al., 2007).

Genetic diversity in plant materials results from variations in DNA sequences and environmental effects. In addition, it is used as a resource for re-vegetation of disturbed sites to allow natural selection and adaptation to occur. Therefore, estimation of the genetic diversity among plants is important for the improvement of any crop and for preserving natural variation for adaptation (Mondini et al., 2009). Genetic diversity can be determined using morphological, biochemical, and molecular markers (Gonçalves et al., 2008). These markers differ from each other with respect to important features such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements, cost, and the type of data that they generate.

Seed storage proteins are deposited in relatively large quantities in mature seeds and typically remain more stable than other plant tissues until they germinate (Mirali et al., 2007). Therefore, proteins can be easily extracted from seeds and analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique leading to separation of seed storage proteins into specific banding patterns, which generates higher levels of genetic polymorphisms on the basis of differences in protein intensity among genotypes (Sinha et al., 2012). Additionally, it is a method commonly used to investigate genetic diversity and to classify plant varieties (Kakaei and Kahrizi, 2011), as genetic markers for genetic variation, to detect genetic diversity in cultivated and wild plant species, and to provide information on phylogenetic relationships among accessions (Kumar and Tata, 2010; Emre, 2011). The major advantages of this protein marker technique include assessments of

codominance, absence of epistatic and pleiotropic effects, ease of use, and a comparatively inexpensive yet powerful method of measuring allele frequencies for specific genes (Mondini et al., 2009).

Molecular markers, particularly DNA genetic markers, are valuable in that they show genetic differences on a more detailed level without interference from environmental influences (Kumar et al., 2009), and involve techniques that provide fast results detailing genetic variation and reflecting underlying genetic diversity (Gonçalves et al., 2008). Furthermore, DNA polymorphisms have become the markers of choice for investigating phylogenetic relationships among various plant varieties (Martosa et al., 2005) and for molecular characterization (Singh et al., 2010). Previous studies evaluated genetic diversity among fenugreek accessions using molecular markers such as rapid amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSRs) (Marzougui et al., 2009; Harish et al., 2011; Sundaram and Purwar, 2011).

All organisms have numerous differences in their genomic DNA sequence and therefore are genotypically distinct. Restricted fragment length polymorphism (RFLP) analysis of nuclear DNA was one of the first techniques to be widely used for detecting variation at the DNA sequence level and can detect a relatively large number of loci scattered throughout the entire genome, thus revealing a high level of polymorphisms that are less influenced by time, space, and other environmental factors (Semagn et al., 2006). Therefore, RFLP has been widely used in studying genetic variation and phylogenetic relationships among populations, species, and varieties (Sun et al., 2001), as fingerprinting tools (Fang et al., 1997), in genetic diversity analyses within and among populations of wild barley (Zhang et al., 1993), as a major tool to identify the genetic diversity within and between species and varieties (Dubreuil et al., 1999), and in the identification of ecotypes (Pupilli et al., 2000). It is now in common use for ecological, evolutionary, taxonomical, phylogenetic, and genetic studies of several plant sciences (Mondini et al., 2009). The major strength of RFLP markers are their high reproducibility, codominant inheritance, good transferability between laboratories and locus-specific markers that allow synteny (conserved order of genes between related organisms). RFLP markers are highly recommended for phylogenetic analysis between related species, as no sequence information is required and they are relatively easy to score due to large size differences between fragments (Semagn et al., 2006).

The present study aimed to: i) compare the usefulness of SDS-PAGE and RFLP for examining levels of genetic variation and polymorphisms among seven fenugreek germplasm accessions from a wide range of geographical origins, and ii) estimate the genetic relationships among these accessions.

MATERIAL AND METHODS

Plant materials

Seeds of seven wild accessions of fenugreek plants (*T. foenum-graecum*) were obtained from the repository of seed bank at King Abdulaziz City for Science and Technology, Saudi Arabia. Plants used in this investigation were collected from diverse ecogeographical areas in Saudi Arabia and Yemen (Table 1). Viable seeds were screened for size uniformity,

and divided into two groups. The first group was subjected to SDS-PAGE analysis, and the second group was used in RFLP analysis. Seeds were used instead of leaves for the SDS-PAGE and RFLP analyses, since this enables accurate estimation of protein and DNA polymorphisms in species with reduced leaves or leaves that accumulate staining inhibitors within leaves, as well as for species growing in regions where these techniques are not readily available (Sliwinska et al., 2009).

Table 1. List of *Trigonella foenum-graecum* accessions used in the present study and their origin.

Serial No.	Germplasm accession No.	Origin
1	122	Saudi Arabia
2	51	Yemen
3	136	Saudi Arabia
4	12	Saudi Arabia
5	50	Yemen
6	98	Yemen
7	74	Saudi Arabia

Biochemical analysis using SDS-PAGE

Preparation of seed cake and defatted meal preparation

Sterilized seeds were milled and defatted according to methods described by Hojilla-Evangelista and Evangelista (2006).

Extraction of seed storage proteins and SDS-PAGE analysis

The protein extraction technique employed was similar to the extraction technique described by Saraswati et al. (1993). Sample buffer was added to 0.2 g seed flour as extraction liquid and mixed thoroughly in an Eppendorf tube by vortexing. The extraction buffer contained the following components (final concentration): 0.5 M Tris-HCl, pH 6.8, 2.5% SDS, 5% urea, and 5% 2-mercaptoethanol. Before centrifugation at 10,000 *g* for 5 min at 4°C, the sample buffer was boiled for 5 min. SDS-PAGE was performed using a standard method on a vertical slab gel. Bromophenol blue was added to the supernatant as a tracking dye to watch the movement of proteins on the gel. Protein profiling of samples was performed using SDS-PAGE as described by Laemmli (1970). Seed proteins were analyzed by SDS-PAGE on 10% polyacrylamide gel. After electrophoresis, the protein bands were visualized by staining with Coomassie brilliant blue G-250. Marker proteins (Fermentas) were used as references. The bands produced in the electropherogram were scored, and their molecular weights were compared to the standard Pharmacia protein marker.

Protein imaging and data analysis

Gel photography and documentation were carried out using the Bio-Rad gel documentation system. The number of bands revealed on each gel lane was counted and compared using the Gel Pro-Analyzer software. Quantitative variations in band number and concentration were estimated using the BIO-RAD video densitometer, Model Gel Doc 2000. With re-

gard to variation in protein banding patterns, electrophoregrams of each germplasm accession were scored for the presence or absence of bands and used to construct a dendrogram by the unweighted pair group method with arithmetic mean (UPGMA).

DNA analysis using RFLP

Isolation of genomic DNA

Genomic DNA from fenugreek seeds was isolated using the hexadecyl trimethyl ammonium bromide (CTAB) method as described by Kit and Chandran (2010).

Qualitative and quantitative analyses of extracted DNA

The DNA yield was measured using a UV-visible spectrophotometer (Perkin Elmer) at 260 nm. DNA purity was determined by calculating the absorbance ratio at $A_{260/280}$ nm. Polysaccharide contamination was assessed by calculating the absorbance ratio at $A_{260/230}$ nm (Wilson and Walker, 2005). For quality and yield assessments, electrophoresis was performed for all of the DNA samples on 0.8% agarose gels, and the gels were stained with ethidium bromide; the bands were observed in a gel documentation system (Alpha Innotech) and compared with the known standard lambda DNA marker.

Digestion and electrophoresis

FastDigest® Restriction Enzymes (*Eag*I, *Eco*RI, *Fsp*I, and *Hin*dIII) were used according to manufacturer instructions to cleave genomic DNA of the plant materials at specific target nucleotide sequences into DNA fragments of different size. The extracted DNA was subjected to RFLP analysis (Parani et al., 1997). Briefly, the reaction mixture was prepared by adding 10 µL extracted DNA, 15 µL 2X assay buffer, 10 µL bovine serum albumin (BSA), and 3 µL of each restriction enzyme. The vials were incubated at 37°C for 1 h for complete digestion. The restriction enzyme-digested products were visualized through silver staining of the polyacrylamide gel. The gels were fixed in 50 mL fixing solution (diluted five times with 30.4 mL double-distilled water and 9.6 mL ethanol) for 30 min and silver-impregnated (with 1X staining solution) for another 30 min. Gels were then washed in double-distilled water for 1 to 2 min. After removing the staining solution, the gels were kept in the developing solution in the dark for 10 min. When the bands were dark enough, the developing solution was poured out, and the stopping and preserving solution was immediately added. At that time, photographs were taken using a digital system and analyzed with computer software.

Scoring and data analyses

The number of DNA fragments revealed in each lane was counted, compared, and regressed to a standard curve of lambda DNA digested with *Hin*dIII, which included fragments from 100 to 4000 bp. Data were scored as the presence or absence of DNA fragments. Gels were visualized with the PhotoPrint (Vilber Lourmat, France) imaging system, and quantitative variations in fragment number and size were analyzed with the Bio-One D+ software (Vilber Lourmat).

Cluster analysis

The PAST computer program (dendrogram window) was used for UPGMA clustering analysis to indicate the genetic relationships among the seven fenugreek germplasm accessions, based on the four restriction enzymes used in the RFLP analysis. Consequently, the presence or absence of DNA fragments and their average was used as an approximate value for estimating genetic relationships in the dendrogram.

RESULTS

SDS-PAGE analysis

Survey of electrophoretic protein polymorphisms

The electrophoretic banding patterns of total seed protein as revealed by SDS-PAGE were used to detect the genetic diversity among seven fenugreek accessions. These results revealed a total of 168 polypeptide bands with different molecular weights ranging from 4.5 to 300 kDa, of which 28 bands were polymorphic with 80.0% polymorphism (Table 2 and Figure 1). The maximum number of bands was found in germplasm accession 122 of fenugreek with a polymorphism value of 19.04%. The minimum number of bands (15) was found in germplasm accession 12 with a polymorphism value of 8.93%. On the other hand, germplasm accession 122 showed two new protein bands, representing unique bands of 15.4 and 5.0 kDa in size, which can be used as markers for this accession. Overall, SDS-PAGE analysis of seed storage proteins revealed three types of bands: monomorphic (bands appeared in all accessions), polymorphic (bands appeared in some accessions and not others), and unique (band appeared in only one accession). These bands varied quantitatively and qualitatively with respect to molecular weight, concentration, relative mobility, or fractionation. Consequently, these bands can be considered as biochemical markers to characterize each germplasm accession. In contrast, several protein bands disappeared in some accessions and appeared in others. The resulting profiles showed different patterns, indicating variability among accessions from different habitats (Figure 1).

UPGMA clustering dendrogram based on SDS-PAGE analysis

Two major clusters were revealed based on the SDS-PAGE data. The first (I) cluster comprised species in germplasm accession 12, which was completely separated from the other six accessions in the second (II) cluster and the genetic relationship ratio was 77.13%. The second (II) cluster was divided into two sub-clusters, (i) and (ii), with a genetic relationship ratio of 79.57%. Sub-cluster (i) comprised fenugreek germplasm in two accessions, 122 and 136, with a genetic relationship ratio of 86.49%, while sub-cluster (ii) comprised two groups, 1 and 2, with a genetic relationship ratio of 82.35%. Group 1 comprised two clades with a genetic relationship ratio of 85.27%, one comprised fenugreek germplasm accessions 51 and 74 with a genetic relationship ratio of 90.32%, while clade 2 comprised fenugreek germplasm of accession 50. On the other hand, group 2 comprised fenugreek germplasm of accession 98. From these results, the two accessions 136 and 74 were shown to be very closely related, followed by accessions 12 and 51 (Figure 2).

Table 2. SDS-PAGE analysis of seed storage protein by documentation system Model (Gel Doc BioRad system 2000 in the lanes 1-7 of studied germplasm accession Nos. 122, 51, 136, 12, 50, 98, and 74, respectively.

Molecular weights of proteins	Number and percentage of protein bands in each sample														Total polymorphic bands				Monomorphic bands	% of monomorphic	% of total polymorphism
	Lane 1	%	Lane 2	%	Lane 3	%	Lane 4	%	Lane 5	%	Lane 6	%	Lane 7	%	Total bands	Unique bands	%	Non-unique	%	% total polymorphic			
300-4.5	32	19.04	19	11.31	27	16.07	15	8.93	22	13.10	25	14.88	28	16.67	168	2	1.19	26	15.48	16.67	7	4.17	80.00

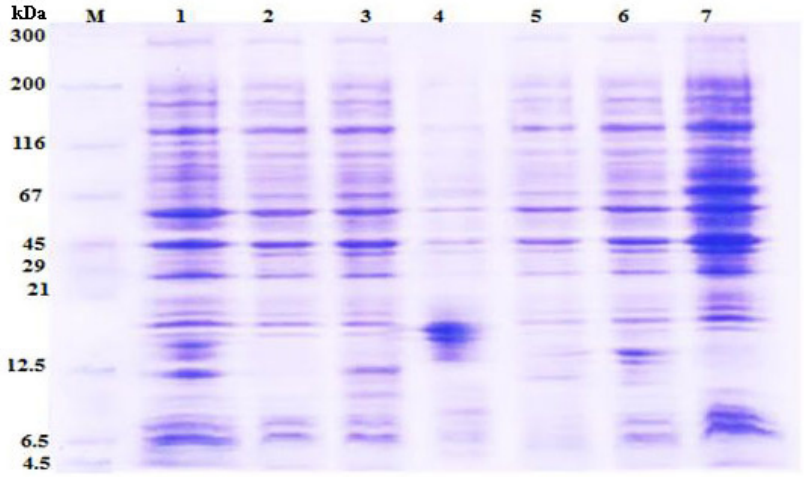

Figure 1. Seed protein banding pattern of studied fenugreek accessions using SDS-PAGE analysis. *Lanes 1-7* = germplasm accessions 122, 51, 136, 12, 50, 98, and 74, respectively. *Lane M* = standard protein marker.

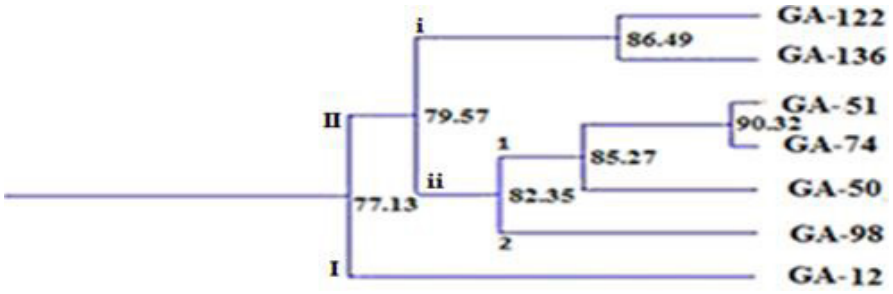

Figure 2. Dendrogram representing genetic relationship between studied fenugreek germplasm accessions (GA) as revealed by UPGMA clustering using simple band matches (tolerance: 3.20%) based on all SDS-PAGE database analysis.

RFLP analysis

Survey of RFLP polymorphisms

Genomic DNA in this study was digested with four restriction enzymes (*Eag*I, *Eco*RI, *Fsp*I, and *Hin*dIII) generating different numbers of DNA fragments. In total, 166 reproducible DNA fragments were scored after using the four restriction enzymes, resulting in a reproducible set of specific DNA fragments with known sequences and variable lengths that were specific for each fenugreek accession on gel electrophoresis. Table 3 shows the total polymorphisms produced by the four restriction enzymes, which reached a value of 88.71%. The restriction enzymes differed with respect to the amount of polymorphisms detected. The highest level of polymorphism among the seven accessions was detected when genomic DNA was digested with *Hin*dIII (100% polymorphism), followed by *Eco*RI (83.33%), *Eag*I (81.82%), and *Fsp*I (70.0%).

Table 3. RFLP products of DNA extracted from seven fenugreek accessions under study generated by restriction enzymes *Eag*I, *Eco*RI, *Fsp*I and *Hin*dIII.

Restriction enzymes	Recognition site sequence	Genomic source	Fragment lengths (bp)	Total DNA fragments generated by restriction enzymes									Polymorphic fragments					Monomorphic fragments	% of monomorphic	% of total polymorphism
				Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Total	%	Unique	%	Non-unique	%	% of total polymorphic			
1-*Eag*I	CGGCCG	*Enterobacter agglomerans*	850-117	6	6	7	5	7	6	4	41	24.70	5	12.20	4	9.76	21.95	2	4.88	81.82
2-*Eco*RI	GAATTC	*Escherichia coli*	800-100	5	7	5	6	5	6	5	93	23.49	-	-	10	25.64	25.64	2	5.13	83.33
3-*Fsp*I	TGCGCA	*Fischerella* species	1000-100	5	3	5	4	5	5	4	31	18.67	2	6.45	5	16.13	29.03	3	9.68	70.00
4-*Hin*dIII	AAGCTT	*Haemophilus influenzae*	4000-80	6	11	7	8	9	9	5	55	33.13	5	9.10	24	43.64	52.73	-	-	100.00
Overall total				22 = 13.25%	27 = 16.27%	24 = 14.46%	23 = 13.86%	26 = 15.66%	26 = 15.66%	18 = 10.84%	166		12	7.23	43	25.90	33.13	7	4.22	88.71

The lanes 1-7 are germplasm accession Nos. 122, 51, 136, 12, 50, 98, and 74, respectively.

Furthermore, the RFLP analysis recognized three types of fragments (polymorphic, unique, and monomorphic), which varied quantitatively and qualitatively in length, concentration, relative mobility, or fractionation on the gel (Table 3). In total, 55 polymorphic fragments were obtained, with a polymorphism value of 25.90%, with the four restriction enzymes. The highest value of polymorphic fragments was 52.73%, which was produced by restriction enzyme *Hin*dIII, while the lowest value was 21.95%, which was produced by restriction enzyme *Eag*I. On the other hand, 12 unique fragments were obtained overall, with a value of 7.23%; five unique fragments with values of 12.20 and 9.10% were produced by restriction enzymes *Eag*I and *Hin*dIII, respectively. Furthermore, a total of seven monomorphic fragments were obtained with a value of 4.22%, which were produced by all four restriction enzymes, although restriction enzyme *Fsp*I produced three monomorphic fragments with a value of 9.68%.

Moreover, of the 166 DNA fragments scored, the maximum number of DNA fragments (27 fragments), with a polymorphism value of 16.27%, was found in germplasm accession 51 of fenugreek. The minimum number of DNA fragments (18 fragments), with a value of 10.84%, was found in germplasm accession 74. Germplasm of accessions 50 and 98 showed similar results of 26 fragments with a polymorphism value of 15.66%. Overall, the DNA fragments produced by RFLP analysis of the seven fenugreek germplasm accessions using restriction endonucleases *Eag*I, *Eco*RI, *Fsp*I, and *Hin*dIII revealed 41, 39, 31, and 55 fragments, respectively. The highest value of DNA fragments produced by restriction enzyme *Hin*dIII was 33.13% with sizes ranging from 117 to 850 bp, while the lowest value was 18.67% produced by restriction enzyme *Fsp*I with sizes ranging from 100 to 4000 bp (Table 3 and Figure 3).

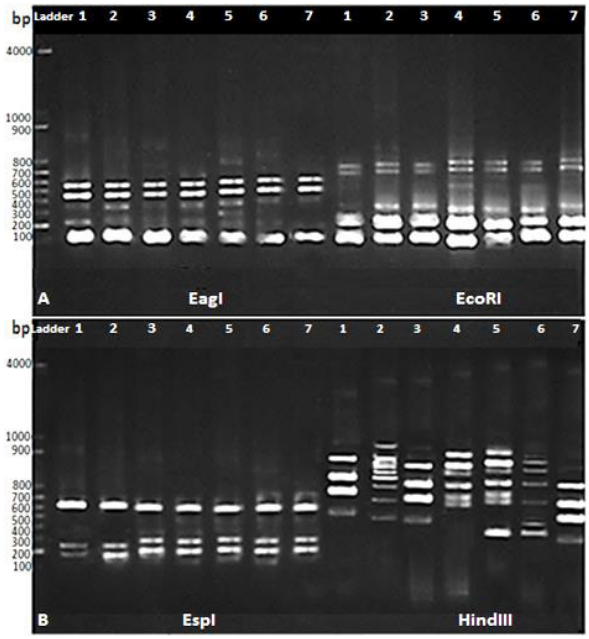

Figure 3. RFLP products of DNA fragments extracted from seeds of fenugreek accessions using restriction enzymes *Eag*I and *Eco*RI (**A**) and *Fsp*I and *Hin*dIII (**B**). *Lanes 1-7* = germplasm accessions 122, 51, 136, 12, 50, 98, and 74, respectively. *Lane Ladder* = 100-bp DNA marker.

UPGMA clustering dendrogram based on RFLP analysis

Two major clusters were obtained from the RFLP data. The first (I) cluster comprised fenugreek germplasm accession 51, which was completely separated from the other six accessions in the second cluster, and the genetic relationship ratio was 56.78%. The second (II) cluster was divided into two sub-clusters (i) and (ii), with a genetic relationship ratio of 71.30%. Sub-cluster (i) comprised two groups, 1 and 2, with a genetic relationship ratio of 74.24%. Group 1 contained two clades, 3 and 4, with a genetic relationship ratio of 78.03%. Clade 3 comprised fenugreek germplasm accession 122, while clade 4 comprised fenugreek germplasm in accessions 12 and 136, with a genetic relationship ratio of 90.91%. Group 2 comprised fenugreek germplasm in accession 74. On the other hand, sub-cluster (ii) comprised fenugreek germplasms in accessions 50 and 98 with a genetic relationship ratio of 77.68%. From these results, two accessions, 12 and 136, were found to be very closely related, followed by accessions 50 and 98, which might have been due to their highly similar polymorphism values (Figure 4).

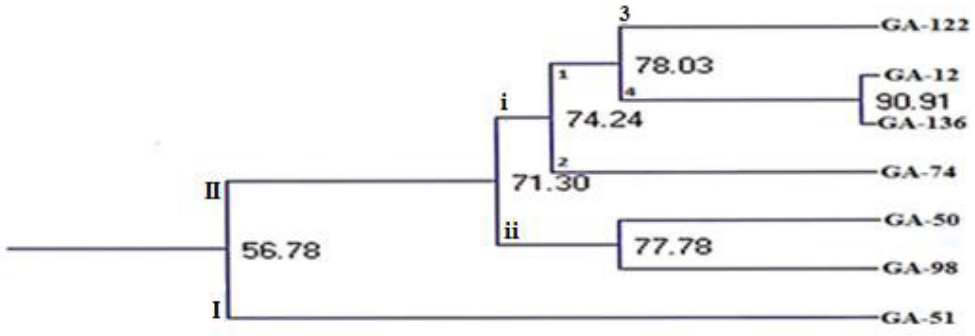

Figure 4. Dendrogram representing genetic relationship between studied fenugreek germplasm accessions (GA) as revealed by UPGMA clustering using simple band matches (tolerance: 3.20%) based on all restriction enzymes of RFLP database analysis.

Comparison of the genetic relationships obtained from cluster analyses based on data from SDS-PAGE or RFLP indicated that the dendrograms showed a clear genetic separation of fenugreek germplasm accessions 12 and 51, respectively, from the other six accessions. However, data based on SDS-PAGE revealed that accession 51 was very closely related to accession 74 with a genetic relationship ratio of 90.32%. Similarly, data based on RFLP revealed that accession 136 was very closely related to accession 12 with a genetic relationship ratio of 90.97%. Moreover, in both analyses, the fenugreek germplasm accessions 50 and 98 were closely related to each other with genetic relationship ratios of 82.35 and 77.78% with SDS-PAGE and RFLP data, respectively.

DISCUSSION

The present study observed that protein distribution patterns of seven fenugreek germplasm accessions (*T. foenum-graecum* L.) revealed qualitative and quantitative intra- and inter-specific variations in terms of band number, staining intensity, and molecular weight. The

resulted profiles showed different patterns, indicating variability among the studied accessions from different habitats. In this respect, Sadia et al. (2009) concluded that differences in electrophoretic profiles are presumably proportional to the genetic specificity of plant species or cultivars because proteins are relatively direct gene products. In addition, the number of different protein variants arising from protein synthesis (alternative splicing and/or post-translational modifications) is significantly greater than the number of genes in a genome, thus it can be considered as markers of these genes. Moreover, seed storage proteins are deposited in relatively large quantities in mature seeds, and typically remain stable until the seed germinates; therefore, they have been used to estimate genetic diversity. Electrophoretic analysis of seed storage proteins provides information concerning the structural genes and their regulatory systems that control the biosynthetic pathways of the protein. In addition, each band represents the final products of transcriptional and translational events occurring due to active structural genes (Srivalli et al., 1999). Therefore, electrophoretic patterns of seed proteins as revealed by SDS-PAGE can be employed for various purposes, such as genetic diversity, biosystematic analysis, and determination of polygenetic relationships and evolutionary relationships of species collected from different natural habitats (Patra and Chawla, 2010; Win et al., 2011).

Protein polymorphism helps in distinguishing plant germplasm at specific levels. Polymorphisms occurring within amino acid sequences may result due to specific environmental factors in different geographical regions (Galani et al., 2011). The data obtained in the present study showed distinct protein polymorphisms in each fenugreek accession on the basis of size, type, and intensity of protein bands, which may result from base changes in DNA altering protein sites. Therefore, these polymorphisms may serve as genetic markers because they can be highly polymorphic and their variability is generally highly heritable. Additionally, protein polymorphisms resulting from insertions or deletions between mutated sites of protein bands are codominant, and these were found in agreement with Mondini et al. (2009). Protein profiling is the most promising tool for determining molecular polymorphisms. Appearance of new bands (unique) usually results from different DNA structural changes (e.g., breaks, transpositions, deletions), which leads to changes in amino acids, and consequently the protein formed (Mondini et al., 2009). The data obtained in the present study also showed that the levels of protein polymorphisms were lower than that of RFLP polymorphisms, which may be attributed to the conservative nature of the seed protein (Bonfitto et al., 1999).

When fenugreek DNA of different accessions was subjected to digestion with four restriction enzymes (*Eag*I, *Eco*RI, *Fsp*I, and *Hin*dIII), the enzyme cleaved the DNA into a greater number of fragments, each defined by the target sequence. Differences in nucleotide sequences at target sites, or in the number of nucleotides between target sites, generates different-sized DNA fragments, which are called RFLPs. High levels of polymorphism generated by RFLP analysis, which is directly targeting the DNA level, reflect heritable changes in the nucleotide sequence, both in coding and non-coding regions. As a result, RFLP studies are more sensitive to genetic changes than are protein studies, which reflect only those changes resulting in specific amino acid substitutions. In this respect, Kumar et al. (2009) concluded that RFLPs show high genomic abundance and random distribution because they reflect restriction size variation that is spread across the genome resulting in the greatest average number of alleles per locus in addition to the frequent occurrence of their recognition sites within genomes. The resulting DNA fragments are examined by electrophoretic separation, and consequently, the presence, absence, or change in the mass of the resulting DNA fragments is evidence of altered DNA sequences. The greater number

of restriction fragments and the higher polymorphism generated by RFLP contributes to the superior discriminative ability of its data and characteristic features (Dubreuil et al., 1999). RFLP analysis of generated DNA fragments is the reflection of structural changes in the whole genomic DNA, and consequently, variation in gene expression. Such polymorphisms can be used to distinguish plant species, genotypes, and, in some cases, individual plants (La et al., 2011). The restriction enzymes used in the present study have revealed the highest levels of polymorphism in previous studies (Nodari et al., 1992).

Variations in the characteristic DNA fragment pattern generated by RFLP analysis of fenugreek in different germplasm accessions may be caused by rearrangements of genomic DNA, base pair deletions, mutations, inversions, translocations, and/or transpositions within restriction enzyme recognition sequences, which result in the loss or gain of a recognition site, ultimately revealing fragments of different lengths, and, thus, polymorphism (Semagn et al., 2006). Fragment sizes of digested DNA will differ depending on the presence (new restriction sites) or absence (removal restriction sites) of the proper recognition sequence for the enzyme, which can be seen after gel electrophoresis. These recognition sites are not associated with any particular type of gene, and are distributed randomly throughout the genome. Since RFLP analysis of nuclear DNA can detect a relatively large number of loci scattered throughout the genome, and the polymorphism revealed by it is less influenced by time, space, and environmental conditions, this technique has been widely used for studying genetic diversity and phylogenetic relationships among populations, closely related species, and varieties (Garcia et al., 2004; Kumar et al., 2009).

In the present study, the germplasm accessions ranged broadly with respect to their relatedness, which may be due to their genetic relationships, diverse ecogeographical areas, or as a result of hybridization or common ancestry. On the other hand, the alterations in DNA band intensities observed among fenugreek germplasm accessions could be interpreted on the basis of alterations of some DNA sequences and of the starting copy number of a particular DNA sequence within the genome. Therefore, the variation in band intensity and disappearance of some bands might correlate with DNA changes due to variations in climatic and environmental factors (Yang and Quiros, 1993).

In conclusion, both SDS-PAGE and RFLP markers were important for estimating genetic diversity among different germplasm accessions of *T. foenum-graecum* L.; however, RFLP analysis showed higher levels of polymorphism than the protein marker. Therefore, protein markers should be augmented with DNA markers for reliable estimates of genetic diversity. On the other hand, genetic relationships resulting from cluster analyses using data from SDS-PAGE or RFLP indicated that these two marker techniques were nearly equivalent, but not identical, with respect to phylogenetic relationships. This reflects the importance of using both marker types in determining genetic relationships between the seven fenugreek genotypes.

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