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Genetic Diversity of Different Varieties of *Foeniculum vulgare* Miller by RAPD Markers

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ABSTRACT

The RAPD technique has been successfully used in a variety of taxonomic and genetic diversity studies. The genetic diversity of seven varieties of Foeniculum vulgare has been evaluated using seven random amplified polymorphic DNA primers. A total of 70 clear bands were generated, out of which 35 (50%) were polymorphic. The total number of markers varied from 4 (GCC-181) to 13 (GCC-90 and GCC-132) with a mean of 10 markers per primer. The number of polymorphic markers for each primer varied from 2 (GCC-181) to 7 (GCC-90, GCC-135 and GCC-176) with a mean of 5 polymorphic markers per primer. The amplified product size ranged from 125 to 3968 bp. The PIC values ranged from 0.081 (GCC-81) to 0.281 (GCC-135), with a mean PIC value of 0.202. The Jaccard's similarity coefficient values ranged from 0.66 to 0.80 with an average of 0.71. A dendrogram constructed based on the UPGMA clustering method, revealed two major clusters. Group-A consisted of two genotypes 1 and 2, where as Group-B could be further classified in to two subgroups Group-I and Group-II. Group-I included genotypes 3, 5 and 6, while Group-II included genotypes 4 and 7. Present study highlights that the high genetic diversity among varieties could be attributed to artificial selection, not natural genetic differentiation.

Keywords: RAPD, *Foeniculum vulgare*, genetic diversity, aromatic, medicinal herb.

INTRODUCTION

Genetic diversity plays a very important role in survival and adaptability of a species because when environment of species changes small gene variations are required to produce changes in the organism's anatomy that enables it to adapt and survive.

Foeniculum is a genus of fewer than half a dozen species, well-known aromatic and medicinal herb which is native to southern Europe and the Mediterranean region. It is carminative and commonly used to flavor liquors, bread, and cheese and in manufacturing of pickles, perfumes, soaps, cosmetics and cough drops [1].

The genetic diversity can be estimated by using morphological, biochemical as well as genetic based tools or advanced molecular methods [2,3]. Morphological markers are less in number and sometimes show epistatic effects. Biochemical markers are also few and may also be influenced by environment and posttranslational modification and their use is very restricted. Molecular markers, being large in number and not affected by environment, are increasingly being used to determine genetic diversity and relatedness among species and varieties [4,5,6]. The most widely used DNA based molecular markers include restriction fragment length polymorphism (RFLP) [7], random amplified polymorphic DNA (RAPD) [8], amplified fragment length polymorphism (AFLP) [9] and microsatellites [10] or simple sequence repeats (SSR). Among these techniques Random Amplified Polymorphic DNA (RAPD) techniques provide very effective and reliable tools for measuring genetic diversity in crop germplasm [11,12,13]. It is simple, quick, and inexpensive method that requires only small amount of DNA. It is a non radioactive based detection assay and does not require any prior sequence information. Hence, it is widely used to study the taxonomy of various genera, species, to differentiate intra-specific variation and to study the genetic diversity of various cultivars and lines [14].

The purpose of the present study was to investigate genetic variation among seven varieties of *Foeniculum vulgare* using RAPD and to characterize these varieties at molecular level that could be utilized for selecting better parents and subsequently provide a base for further strengthening the *Foeniculum* breeding program. The information, thus secured, could be used as a tool to help the conventional breeding for the quality improvement of *Foeniculum.*

MATERIALS AND METHODS

Plant material

Seven varieties of *Foeniculum vulgare* (FNL-41, FNL-46, RF-101, RF-125, RF-143, RF-178, RF-205) were obtained from the Department of Plant Breeding & Genetics, SKN College of Agriculture, Jobner, Rajasthan Agricultural University, Bikaner.

Young leaves without necrotic areas or lesions of each variety were collected and stored overnight in absolute alcohol.

DNA isolation and purification

Genomic DNA was extracted from the young leaves by using Doyle and Doyle method [15]. 4 g of tissue material was ground in absolute alcohol with the help of mortar-pestle. The homogenized material was transferred to 20 ml pre-warmed (60^0C) DNA Isolation Buffer (2X CTAB DNA Extraction Buffer - 100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 2 % CTAB and 2 µl/ml β-mercaptoethanol) in capped polypropylene tubes. It was then incubated for 1 hr. at 60^0C with occasional mixing by gentle swirling in water bath. After removing from water bath one volume of chloroform: Isoamyl alcohol (24:1) was added and mixed by inversion for 15 minutes to ensure emulsification of the phases then spun at 15,000 rpm for 15 minutes (Eltec centrifuge).

Aqueous phase was taken and transferred to another tube. Ice cold 2 volume of absolute alcohol or 0.6 vol. of isopropanol was added to precipitate DNA. DNA-CTAB complex was precipitated as a fibrous network and amorphous precipitation was collected by the centrifugation at 5,000-10,000 rpm for 5-10 minutes at 20°C. 20 ml of 70% alcohol was added to the pellet of DNA and was kept for 20 minutes with gentle agitation. The pellet was collected by centrifugation at 5,000 rpm for 5 minutes at 20°C. The tubes were inverted and drained on a paper towel. The pellet was dried over-night after covering with parafilm with tiny pores. The pellet was re-dissolved in 1000 µl of TE buffer by keeping over night at 4°C without agitation. RNA was removed by treating the sample with RNase. 2.5 µl of RNase was added to 0.5 ml of crude, DNA preparation (2.5 µl of RNase = 25 µg of RNase, so treatment was 50 µg/ml of DNA preparation). Gently it was mixed thoroughly and was incubated at 37°C for 1 hr. Protein including RNase was removed by treating with chloroform: Isoamyl alcohol (24:1).

For the determination of DNA quality and concentration of DNA samples, samples were run in 0.8 % agarose gel formed in 0.5X TBE (Tris Borate EDTA) buffer containing 0.5 µg/ml of Ethidium Bromide. Quantitation of DNA was done by observing it at 260 nm and 280 nm wavelengths by using a UV- VIS spectrophotometer. Optical density ratios were evaluated and only good quality DNA samples were used in polymerase chain reaction.

RAPD analysis

Table 1. RAPD polymorphism among 7 *Foeniculum vulgare* genotypes with primer sequence information.

S.No.	Primers	Sequence	Total no. of bands	No. of polymorphic bands	Polymorphism (%)	Product size (bp)	PIC
1	GCC-60	TTG GCC GAG C	8	5	62.5	125-2122	0.244
2	GCC-81	GAG CAC GGG G	12	4	33.33	350-2200	0.081
3	GCC-90	GGG GGT TAG G	13	7	53.84	125-3968	0.219
4	GCC-132	AGG GAT CTC C	13	3	23.07	125-1500	0.106
5	GCC-135	AAG CTG CGA G	9	7	77.77	510-2100	0.281
6	GCC-176	CAA GGG AGG T	11	7	63.63	350-2310	0.259
7	GCC-181	ATG ACG ACG G	4	2	50	125-950	0.224

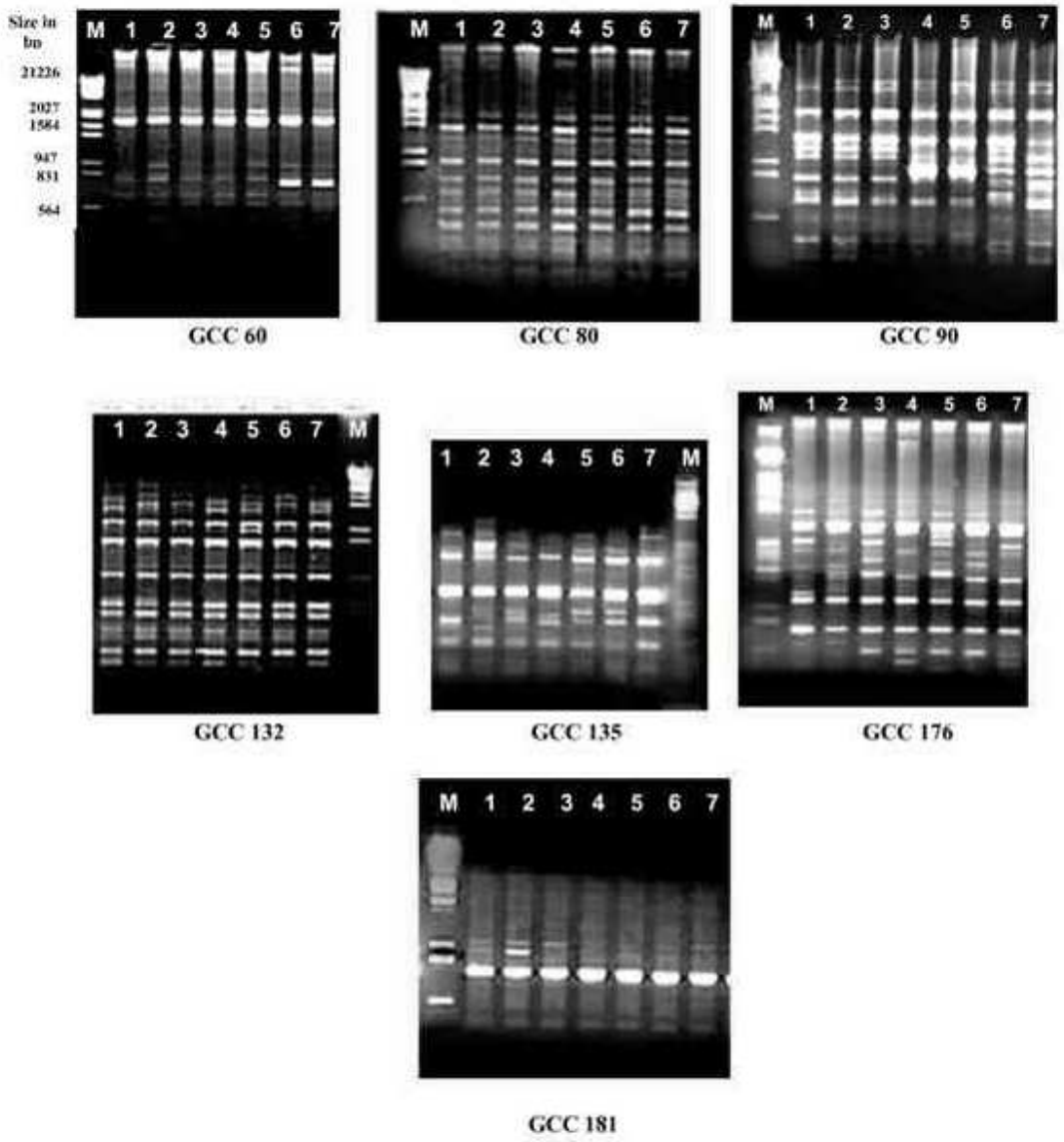
The quantitated DNA was diluted to final concentration of 25 ng/µl in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) for RAPD amplification. Random amplified polymorphic DNA (RAPD) analysis was performed with decamer GCC Primers (see Table 1). PCR reactions were performed in final volume of 25 µl containing 10X Assay Buffer (Bangalore Genei), 1.0 unit of Taq DNA polymerase (Bangalore Genei), 200 µM each of dNTPs (Fermentas), 10 pmols / reaction of random primers and 50 ng of template DNA. The PCR was performed in 'Biometra Thermocycler'. The PCR program comprised 44 cycles and the PCR tubes were subjected to the thermal profile. Setting of the PCR program was based on three steps. Step one was initial denaturation at 94ºC for 5 min. Step two comprised running for 44 cycles, each starting with denaturation at 94ºC for 1 min. followed by annealing 42ºC for 1 min. and ended by extension at 72ºC for 1 min. Step three was a final extension cycle performed at 72ºC for 7 min. The PCR machine was adjusted to hold the product at 4ºC.

Following the amplification, the PCR products were loaded on 1.2 % Agarose gel (Himedia, molecular grade), which was prepared in 1X TBE buffer containing 0.5 µg/ml of the Ethidium

Bromide. The amplified products were subjected to electrophoresis for 3 - 3.5 hrs at 50 V and 100 V, respectively, with cooling. After separation the gel was viewed under UV trans-illuminator (Biometra gel documentation system).

RAPD profile analysis

Figure 1. RAPD profile of *Foeniculum vulgare* varieties obtained with GCC- 60, 80, 90, 132, 135, 176 and 181 primers.



The screened primers that gave bands were used to amplify the DNA of all the 7 fennel varieties. Each genotype was characterized by its banding pattern (Fig. 1). RAPD bands as viewed from the gels after electrophoresis and staining were designated on the basis of their molecular sizes (length of polynucleotide amplified). λ DNA EcoR I/Hind III double digest was loaded with each primer products to estimate the molecular size. These RAPD markers were converted into a

matrix of binary data, where the presence of the band corresponded to value 1 and the absence to value 0. The scores (0 or 1) for each band were entered in the form of a rectangular data matrix (qualitative data matrix). The pair-wise association coefficients were calculated from qualitative data matrix using Jaccard's similarity coefficient.

Cluster analysis for the genetic distance was then carried out using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method. The genetic distances obtained from cluster analysis through UPGMA were used to construct the dendrogram, depicting the relationships of the genotypes using computer program NTSYS pc version 2.02 [16].

Diversity for each marker was determined using the polymorphic information content (PIC), calculated according to [17] as,

$$PIC = 1 - \Sigma pij^2,$$

Where, *pij* is the frequency of the patterns (*j*) for each marker (*i*).

RESULTS

Molecular characterization

The CTAB extraction protocol successfully yielded DNA (167-296 ng/µL) from all the fennel varieties with a A_{260}/A_{280} ratio of 1.7– 1.9. Out of 30 primers screened, most polymorphisms were obtained with seven primers GCC- 60, GCC-81, GCC-90, GCC-132, GCC-135, GCC-176, and GCC- 181 (Table 1).

A total of 70 amplicons were obtained, out of which 35 were polymorphic. The total number of markers varied from 4 (GCC-181) to 13 (GCC-90 and GCC-132) with a mean of 10 markers per primer (Table 1). The number of polymorphic markers for each primer varied from 2 (GCC-181) to 7 (GCC-90, GCC-135 and GCC-176) with a mean of 5 polymorphic markers per primer. The amplified product size ranged from 125 to 3968 bp. The PIC values ranged from 0.081 (GCC-81) to 0.281 (GCC-135), with a mean PIC value of 0.202.

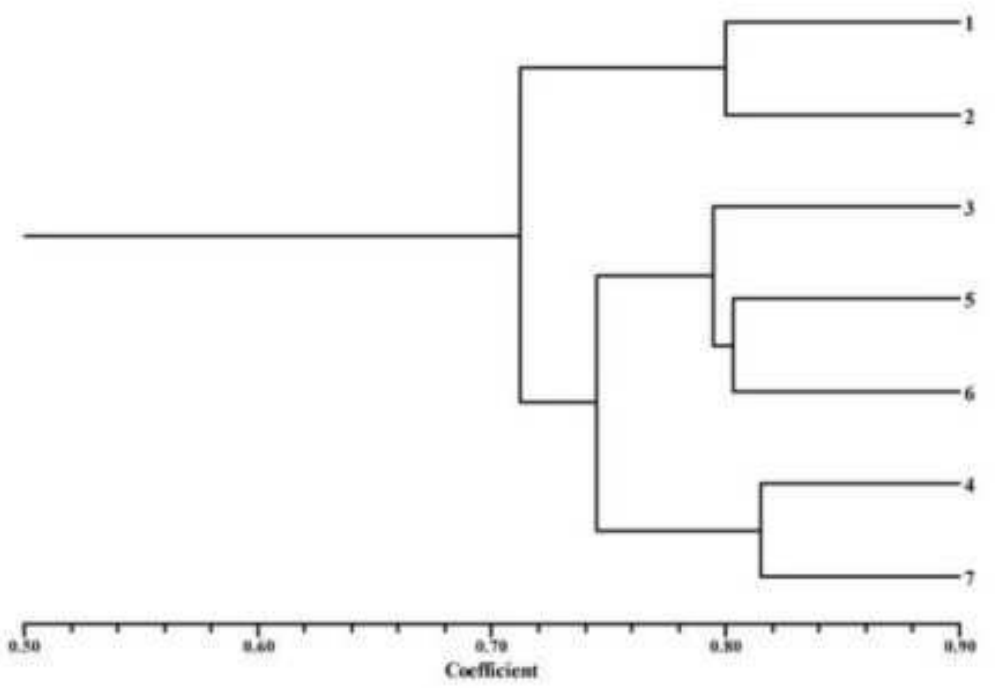
Genetic relationship among the accessions and cluster analysis

Table 2. Jaccard's similarity coefficient 1 to 7 are code for different varieties ; 1-FNL-41; 2-FNL-46; 3-RF-101; 4- RF-125; 5- RF-143; 6-RF-178 and 7- RF-205.

	1	**2**	**3**	**4**	**5**	**6**	**7**
1	1.00						
2	0.80	1.00					
3	0.76	0.77	1.00				
4	0.68	0.67	0.74	1.00			
5	0.69	0.70	0.80	0.75	1.00		
6	0.66	0.75	0.79	0.69	0.80	1.00	
7	0.71	0.75	0.77	0.81	0.75	0.78	1.00

Genetic similarity estimates based on RAPD banding patterns were calculated using method of Jaccard's coefficient analysis (Table 2). The Jaccard's pairwise similarity coefficient values ranged from 0.66 (6 and 1) to 0.80 (2 and 1, 5 and 3, 6 and 5) with an average of 0.71, for single primer based RAPD patterns.

Figure 2. Dendogram showing relationship among seven Fennel genotypes generated by UPGMA analysis based on single primers



The clusters constructed through NTSYS (2.02 pc) presented in the form of dendrogram are shown in Fig. 2. The cluster analysis revealed two major Groups, Group-A and Group-B. Group-A consisted of two genotypes 1 and 2, where as Group-B is further classified in to two subgroups Group-I and Group-II. Group-I included genotypes 3, 5 and 6, while Group-II included genotypes 4 and 7. Group A and B showed 71.4 % between group similarity. Group A comprised two genotypes and showed 80 % within group similarity and Group B comprising two subgroups I and II showed 44.8 % within group similarity.

DISCUSSION

RAPD technique is a common and well-proven tool in genetic studies and a convenient procedure for detecting total genetic variation and it's partitioning within and among populations. The RAPD procedure has been effectively used in a variety of taxonomic and genetic diversity studies [18,19,20]. The simplicity of technique facilitated its use in the analysis of genetic relationship in several instances [21]. The major concerns pertaining RAPD generated phylogeny include homology of bands exhibiting the identical rate of migration causes of variation in fragment mobility and origin of sequence in the genome. Despite these simulations, RAPD marker has distinct advantage in its ability to scan across all regions of the genome, hence

highly appropriate for phylogenetic studies at species level [22]. In the current investigation, the marker technology was employed to detect genetic variation within *F. vulgare* varieties.

F. vulgare showed a high percentage of genetic polymorphism of 50 %, which is close to the percentage for *Changium smyrnidoies* (69 %) [23] but higher than that of *Dacydium pierrei* (33.3 %) [24] and *Cathaya argrophylla* (32 %) [25]. Similarly, the genetic diversity index was also highly variable from 0.54 to 0.73 in case of *F. vulgare* varieties.

Zahid [26] described genetic diversity of 50 indigenous fennel germplasm accessions in Pakistan. These workers used 30 RAPD primers, 24 primers generating a total of 145 bands. Of these 48 % fragments were polymorphic in one or the other DNA amplified profiles. Our data based on indigenous varieties reveal percentage of polymorphism within the range described by Zahid [26] from Pakistan. This also conforms the data for other aromatic plants e.g. *Ocimum gratissimum* [27]. 50 % of bands were monomorphic for the present seven fennel varieties analyzed. Zahid [26] reported 52 % bands as monomorphic in their investigation. The variability could be attributed to differences in the primers used or sequence and the extent of variation in specific genotypes. These could be the reasons for the number of bands in different accessions and / or varieties.

Present studies demonstrate that RAPD is suitably informative and potent enough to evaluate genetic variability of *Foeniculum* varieties. In summary RAPD markers provide a useful tool in planning and execution of germplasm conservation. The primers could be fruitfully exploited for determining genetic variation of *Foeniculum* varieties.

The genetic diversity of the plants is closely related to their geographic distribution. Present RAPD analysis showed high genetic diversity in *F. vulgare* varieties. This may be explained on the basis of selection or interspecific hybridization.

In order to determine genetic diversity among seven varieties of indigenous *Foeniculum*, cluster analysis was done (Table 2). From dendrogram (Fig. 2) it is evident that the different varieties could be divided into two sub clusters genetically at 75 % distance. Cluster-A comprised 2 varieties, FNL-41, FNL-46 were close to each other and their genetic distance is only 5 %. It may be added that the feasibility of naturally occurring genetic cross and gene flow should be high among varieties growing adjacent to each other. Present study highlights that the high genetic diversity among varieties could be attributed to artificial selection, and not natural genetic differentiation.

The level and distribution of genetic diversity detected by RAPD are in overall agreement with recent studies in India [22,28,21,29,30,31]. RAPD, being a multi-locus marker with the simplest and fastest technique, has been successfully employed for the determination of intra-species genetic diversity in several plant species [21]. In *F. vulgare* 1 and 2 samples did not group with any other variety in dendrogram indicating its genetic distinctness from other varieties sampled in our study.

PIC determines the degree of polymorphism of marker, which really is the proportion of individuals that are heterozygous for a marker. In fact PIC is a realistic measure of the

heterozygosity. The calculated PIC (0.224, 0.081, 0.219, 0.106, 0.281, 0.259, and 0.224) based on the probability that two unrelated genotypes amplified from the test population will be placed into different typing groups. So to say it is an index to determine how many alleles a certain marker has and in what way those alleles divide. High PIC value indicates enormous heterozygosity which in turn is associated with a high degree of polymorphism [32]. Thus, varieties RF-143, RF-178 and RF-205, exhibit high heterozygosity.

In *F. vulgare*, good range (0.081-0.281) of PIC value was observed, which indicated significant genetic diversity among *F.vulgare* varieties.

CONCLUSION

Identification of inter-varietals diversity is an essential condition for the analysis of genetic diversity. The present findings unarguably suggest extending the scope of collection of *F. vulgare* to detect and quantify the prevalent genetic diversity existing within different indigenous varieties of *F. vulgare* at the molecular level. To our knowledge this is the first report on the characterization of *F. vulgare* varieties based on available primers from India. RAPD appears to have the potential to distinguish closely related varieties based on their patterns of their amplicons. The level and distribution of genetic diversity detected by RAPD are in overall agreement with recent studies in India. Present study highlights that the high genetic diversity among varieties could be attributed to artificial selection, and not natural genetic differentiation.

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