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RAPD MARKERS BASED GENETIC DIVERSITY OF SAFFLOWER (*CARTHAMUS TINCTORIUS* L.) GERMPLASM

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Abstract

Genetic diversity is extremely significant for the new crop varieties development with elevated yield and other advantageous characters. Molecular level genetic diversity amongst 12 safflower (*Carthamus tinctorius* L.) accessions was studied via Random Amplified Polymorphic DNA (RAPD) markers. Genomic DNA was extracted and optimized PCR protocol for amplification with 08 random primers which produced an entirety 80 amplified fragments with an average polymorphic loci percentage 71 (88.7%). Each primer produced 6 to 13 bands with standard of 10 bands for each primer. Pair wise similarity indices vary from 0.50-0.80 amongst the 12 accessions. Accessions '16337' and '26778'showed the highest similarity index of 80%, whereas the most varied accessions were '26789' and '26748' (50%). All accessions were grouped into three main clusters based on Un-weighted Pair Group Method with Arithmetic averages (UPGMA). Accession number 26753 was different from rest of the safflower accessions which made a separate cluster. In conclusion, even we used only eight molecular primers for this genetic diversity assessment, but showed good result. Our excremental work will be highly supportive for the conservation and improvement of safflower germplasm for the future research and cultivation of this important oil yielding crop in the country.

Key Words: Genetic diversity, RAPD markers, Safflower germplasm

Introduction

Safflower (*Carthamus tinctorius* L.) as compared to other oil crops is a minor crop worldwide. It belongs to the family Compositae or Asteraceae. Artichoke, Chrysanthemum, Niger and sunflower are other members of the family (Yuan *et al.,* 1989). Among safflower species only *Carthamus tinctorius* L. containing 12 pairs of chromosomes are grown worldwide (Kumar *et al.,* 1981). Both Africa and Asia are considered as its origin and Mediterranean as the main production area (Rapoti & Romvari, 1972).

In India and Pakistan, it is most commonly known as 'kusum' which is derived from the Sanskrit, 'kusumbha' (Chavan, 1961). It is an annual, broadleaf oilseed crop. In the northern and southern hemispheres, between the latitudes of 50 degrees and 23 degrees with a cycle of 110 to 150 days to mature is grown in most agricultural areas in the world. Maturity of safflower germplasm depends on day length (Gilbert *et al.,* 2008). Maas & Hoffman, (1977) categorized safflower as moderately salt tolerant crop but its yield is substantially reduced due to high soil salinity (Rains *et al*., 1987). As compared to majority of the crops, water requirement of safflower is limited. Even if the water supply at the reproductive stages is low the most favorable yield of safflower seed can be achieved (Katara & Bansal, 1995). Its fruits or seeds also contain a variety of polyphenolic composites like lignans glucosides, serotonins and flavonoids. Their chemical arrangement and antioxidant properties have been characterized as phytoestrogens with weak estrogenic or anti-estrogenic properties towards mammals. Yellow (yellow 'A') and red quinochalcone stains are present in safflower petals (Takahashi *et al*., 1982). The whole plants (flowers, seeds, and oil) have a wide range of medicinal uses. Flowers are used in Chinese herbal planning against chronic diseases (Dajue & Mundel, 1996). For the prevention of abortion and infertility in women different countries like India and Afghanistan used as 'tea' from its foliage (Weiss, 1983).

Safflower seed contains 25-37% oil and have been found in 4,000 year-old Egyptian tombs. Its use was recorded in China approximately 2,200 years ago (Vincent & Jimmerson, 2005). Three main products come from current safflower production i.e. oil, meal, and birdseed. The primary product is seed oil which constitutes 30 to 45 percent as food and industrial usage. Safflower has two types of oil; Oleic oil which has high monounsaturated oleic fatty acid and linoleic oil having high polyunsaturated linoleic fatty acid. Oleic oils are used for cooking while linoleic oil is utilized as drying agent in paints and varnishes. Seed extracts have been used for the treatment of blood continuity, osteoporosis and endorsement of bone development (Rodan & Martin, 2000). Seeds might be used as a drug for the renewal of bones (Seo *et al*., 2000). In Pakistan, about 362 accessions are present at the gene bank of NARC Islamabad and mainly cultivated in Sindh and Baluchistan. In 2008-09, its national average yield was 890 kg/ha (www.parc.gov.pk). The Food and Agriculture Organization (Anonymous, 2010) estimated the world safflower production at approximately 600,000 ton in India being foremost, exceeding more than double that of any other country. In Pakistan and Bangladesh seed decoctions are used as laxative with sugar and to lessen rheumatic pains respectively. In Kashmir it is used with mustard oil for flush out of urinary tracts.

Safflower is grown in around 60 countries around the world. About half of the world's safflower production per year is reported in India (Gyulai, 1996). Safflower has a major role in farming system although it is believed to be a minor crop with less than 1 million hectares planted, producing around 500,000 metric ton per year. India is the leading producer of safflower followed by USA as second major producer in the world (Gilbert *et al.,* 2008).

Genetic diversity plays a key role in the crop improvement and needed among all correlated varieties, and required for crops evolutionary history and genetic resources (Zada *et al.,* 2013)

RAPD primers are extensively used for germplasm categorization and genetic diversity evaluation (Shinwari *et al.,* 2013), gene tagging, in genetic purity testing and also for the identification of cultivars (Rabbani *et al.,* 2010). Relatively low cost and high speed is an asset of RAPD markers for evaluation of genetic diversity of different crops (Williams *et al*., 1990). RAPD markers have high resolving power, easier to handle and better quality results, as compared to other conventional markers such as RFLP. Theses markers have proved their results in various economical crops such as, rice (Rabbani *et al*., 2008; Pervaiz *et al*., 2010), sesame (Akbar *et al*., 2011) and horticultural plants similar to common bean, strawberry, neem, turmeric (Jan *et al*., 2011) etc. In the present research work different safflower germplasm were evaluated via RAPD genetic markers. Our work will be helpful to a great extent in the documentation, classification and conservation of different safflower accessions for the better and improved varieties in the country.

Materials and Methods

Plant material: Twelve accessions of safflower were selected, based on morphological characters for RAPD analysis (Table 1). About twenty seeds samples of safflower were sown in small pots under greenhouse condition. After two weeks of plantlets growth fresh leaves were collected for DNA extraction.

DNA extraction and PCR analysis: With the help of a standard CTAB DNA extraction protocol (Doyle & Doyle, 1990), DNA was extracted from all the safflower genotypes. The extracted total genomic DNA of all the accessions was diluted to a working concentration with TE buffer (20ng/µl) for further PCR analysis. About 35 different RAPD primers (Operon Technologies Inc. Alameda, California, USA) were used for our experiment and eight primers were screened on the basis of good result. After the screening process, 8 primers with clear and consistent banding pattern results were chosen for further molecular analysis. Final volume of amplification reactions made 20µl. Composition of reaction mixture was 50mM KCl], 1.9mM MgCl2, 0.26mM dNTP, 1x PCR buffer [10mM Tris-HCl (pH 8.3) and 0.5mM of 10-mer primer (Operan Technologies Inc., Alameda, CA), one unit Taq DNA polymerase enzyme and 20ng of total genomic DNA. Polymerase chain reaction process started with one cycle of five minutes at temperature 94^{o}C for first strand separation of DNA, 45 cycles at 94^{o}C for 1 minute (denaturation), one minute at 36^{o}C temperature for DNA double strand annealing, with two minute for extension of primer. At the end one cycle of seven minute at 72^{o}C for the final extension at a low temperature. Finally, about 10µl of amplification mixture with loading dye was loaded in 1% agarose for gel electrophoresis in 1xTBE buffer (10mM Tris-Borate, 1mM EDTA) for PCR products analysis. We used 1kb DNA ladder as a standard molecular marker. After gel electrophoresis completion, stained with Ethidium bromide for a period of 45 minutes and took pictures under UV light (black and white film # 667 -Polaroid, Cambridge, Mass., USA).

Table 1. List of safflower germplasm used for RAPD analysis.

S. No.	Accession	Origin	Locality
1	16282	Iran	_
2	16337	Afghanistan	_
3	26737	USA	Arizona
4	26748	USA	Idaho
5	26753	China	Beijing
6	26775	China	Beijing
7	26778	USA	California
8	26789	China	--
9	26797	Not mention	_
10	26798	Not mention	_
11	26799	Uzbekistan	_
12	26800	Afghanistan	_

Data analysis: After PCR amplification data was scored on the basis of presence and absence of DNA bands i.e. absence (0) and presence (1). There were many bands with high intensity but we did not considered it and all DNA fragments with the same mobility were scored as the same DNA band. Only main DNA bands were considered for further statistical analysis. Molecular marker of 1kb known size was used in the experiment. Pair-wise similarity of all the safflower genotypes on the basis of absence and presence of exclusive and mutual DNA bands were employed to mark similarity coefficients. Assessments of genetic comparison (F) calculated between all pairs of safflower germplasm via Dice algorithm. The Dice algorithm is just like that of Nei & Li, (1979) as follows:

Similarity (F) = 2Nxy / (Nx + Ny), Where

Nx = Number of DNA bands noticed in individual ‘x’,

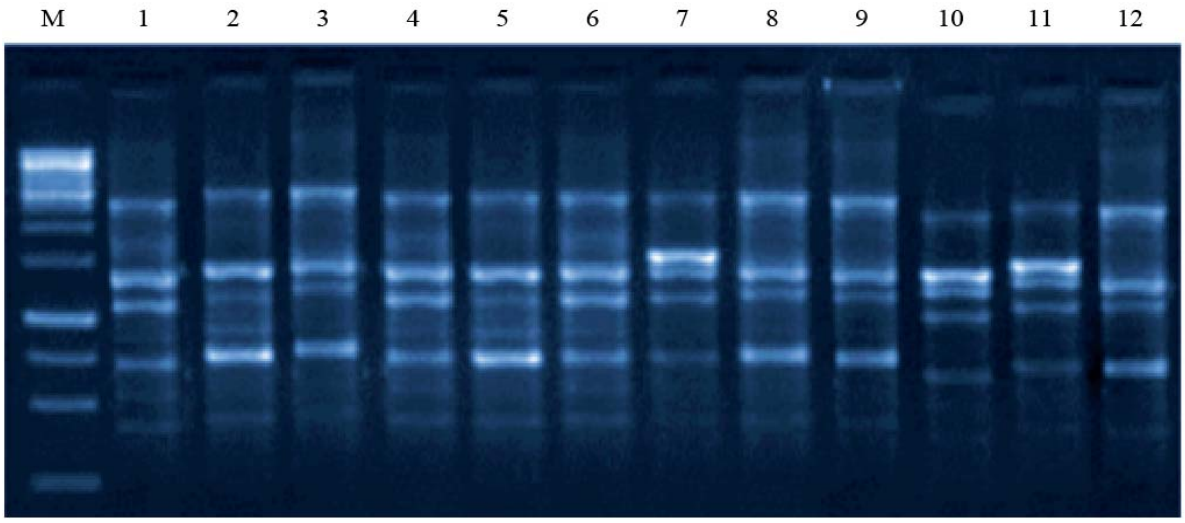
Ny = Total bands shown by individual ‘y’ and

Nxy = Bands shared by individuals ‘x’ and ‘y’

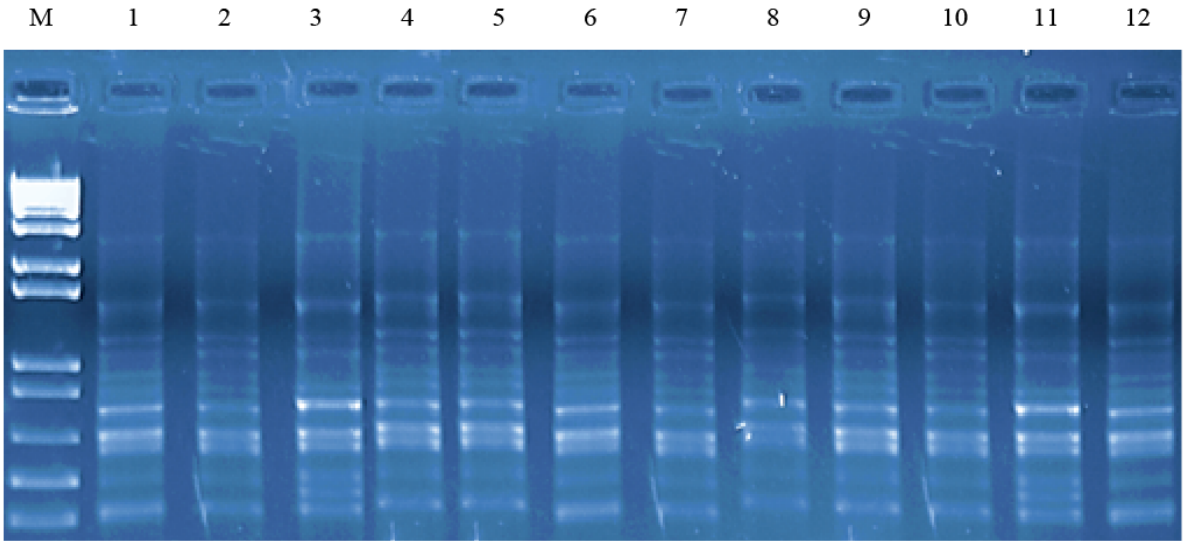
Results and Discussion

A total of twelve safflower accessions were evaluated via RAPD markers which produced various bands after PCR amplification (Fig. 1). Primers OPA-10 and OPA-16 gave maximum polymorphism i.e., 100%, while minimum polymorphism was shown by OPA-09 (66%), OPA-18 and OPB-05 (83%). As whole all the amplified primers showed high level of polymorphism. Most of the bands were similar but some accessions showed characteristic DNA bands pattern and were different from rest of the bands produced by different primers. Some accessions showed high number of bands, while other accessions showed least number of DNA bands. High number of bands was revealed in accession, '16337' which gave 48 bands, while two accessions '26753 and 26775' gave 45 DNA bands each. Some of the primers (OPA-03, OPA-04, OPA-10 and OPA-16) generated widespread amplified products furthermore elevated intensity of genetic assortment, whereas least bands (six bands) plus slight randomness was generated by the primers like OPA-05 and OPA-18.

A total of 80 bands were generated as a result of 08 primers in all 12 accessions, out of eighty DNA bands 71 (88.7%) bands were polymorphic (Table 2). While overall polymorphism ranged from 66.6 to 100%. Detail of all the primers and bands of all accessions was such that OPA-01 produced thirteen bands and eleven were polymorphic (84.6%), 0PA-03 gave total nine bands and all except one band were polymorphic (88.8%), OPA-04 like the above primer produced nine bands and in these band only one band was monomorphic, while rest of the bands were polymorphic in nature (88.8%). Similarly other primers also produced mixed polymorphic and monomorphic bands (Table 2).



OPA-01



B. OPA-09

Fig.1. Amplification of safflower accessions by RAPD markers OPA-01 and OPA-09. M = 1kb DNA ladder, 1 = 16282, 2 = 16337, 3 = 26737, 4 = 26748, 5 = 26753, 6 = 26775, 7 = 26778, 8 = 26789, 9 = 26797, 10 = 26798, 11 = 26799, 12 = 26800.

Table 2. Primers used for generating RAPDs in safflower genotypes.

S. No.	Prime names	Sequence (5'-3')	Amplified fragments (a)	Polymorphic fragments (b)	Percentage polymorphism (bx100/a)	Fragment size range (bp)
1	OPA-01	CAGGACCCTTC	13	11	84.6%	350-1kb
2	OPA-03	AGTCAGCCAC	9	8	88.8%	250-3000
3	OPA-04	AATCGGGCTG	9	8	88.8%	270-2000
4	OPA-09	GGGTAACGCC	12	8	66.6%	350-4800
5	OPA-10	GTGATCGCAG	13	13	100%	250-2000
6	OPA-16	AGCCAGCGAA	12	12	100%	500-1kb
7	OPA-18	AGGTGACCGT	6	5	83.3%	250-2500
8	OPB-05	TGCGCCCTTC	6	5	83.3%	375-1000
Total			**80**	**71**	**88.7%**	

Table 3. Dice coefficients of similarity among 12 safflower genotypes.

Accession	16282	16337	26737	26748	26753	26775	26778	26789	26797	26798	26799	26800
16282	1.00											
16337	0.63	1.00										
26737	0.58	0.71	1.00									
26748	0.70	0.56	0.55	1.00								
26753	0.64	0.73	0.71	0.56	1.00							
26775	0.68	0.64	0.62	0.74	0.61	1.00						
26778	0.72	**0.80**	0.67	0.63	0.64	0.62	1.00					
26789	0.63	0.77	0.76	**0.50**	0.73	0.67	0.71	1.00				
26797	0.61	0.76	0.70	0.61	0.69	0.77	0.70	0.70	1.00			
26798	0.68	0.71	0.64	0.60	0.63	0.62	0.74	0.57	0.73	1.00		
26799	0.63	0.71	0.74	0.55	0.61	0.64	0.74	0.64	0.74	0.79	1.00	
26800	0.65	0.67	0.62	0.60	0.61	0.64	0.69	0.59	0.68	0.78	0.74	1.00

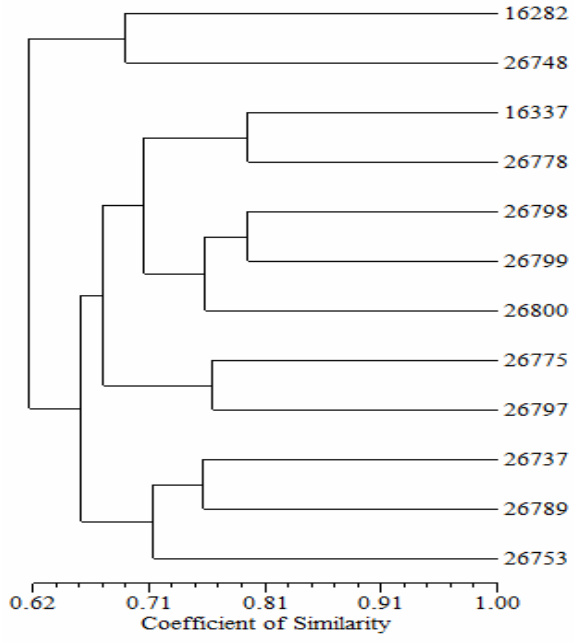


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Fig.2. UPGMA cluster analysis showing diversity among 12 safflower accessions.

To estimate the similarities among different safflower accessions principal component analysis was used. Pair-wise estimates of similarity for all 12 accessions varied from 0.50 to 0.80 (Table 3). The highest similarity index (80%) showed by accessions '16337' and '26778', while two accessions '26789' and '26748' revealed least similarity index (50%). All accessions showed some differences among one another.

On the basis of Nei and Li's similarity matrix via UPGMA analysis, 12 safflower accessions were grouped into three main groups i.e., 'I, II and III (Fig. 2). Further groups I and II were subdivided into IA and IB, IIA, and IIB, respectively. The biggest group was Group II in which the first biggest subgroup of the dendrogram was cluster IIB with 5 accessions, 26800, 26799, 26798, 26778 and 16337. All these genotypes were gathered at similarity coefficient of 0.65. Second biggest group in dendrogram was group I with 3 accessions one in subgroup IA (26753) and two in subgroup IB (26789, 26737). Group III had two accessions, 26748 and 16282. The similarity coefficient among these accessions shown in dendrogram ranged from 0.62 to 0.1.

From the cluster analysis it is revealed that all the safflower accessions were far apart from one another sowed least similarities. High degree of polymorphism

were observed in the 12 genotypes of safflower using eight RAPD markers generated 80 amplified fragments with average percentage of polymorphic loci 71 (88.7%). Our results were supported by Safavi *et al*., (2010) who used 13 RAPD primers generating 74 amplified fragments in which 60 were polymorphic showing related polymorphism i.e. 81.08 %. Mahasi *et al.,* (2009) and Khan *et al.*, (2009) found out similar results to our present findings generating 61 and 78 amplified fragments from 14 and 15 RAPD primers, respectively. On safflower same work reported by Amini *et al.,* (2008), and Souframanien & Gopalakrishna, (2004) used 15 and 25 RAPD primers which produced total polymorphic bands 132 and 44, respectively.

Variation in amplified products (6-13 bands with standard of 10 bands for each primer) were examined in present work is in an agreement with Khan *et al*., (2009), Amini *et al.,* (2008) and Safavi *et al*., (2010) findings, who noticed 1-10 and 3-16 and 3-9 RAPD fragments generated per primer with an average of 5.5, 9.5 and 6 bands in safflower, respectively. Souframanien & Gopalakrishna, (2004) and Panahi *et al*., (2013) work is also in support of our results calculated amplified products range from 3 to 9 and 8 to 18 through standard of 1.8 and 13 bands for each primer. All of the conclusions are hold up by Kernodle *et al*., (1993) and Devos & Gale, (1992) evidence that the difference in the quantity of amplified bands by diverse primers is affected by means of a series of aspects such as sensitive working atmosphere, structure of primer, genome least numbers of annealing sites and quantity of template used.

Pair-wise similarity of our study among the 12 accessions of safflower varied from 0.50 to 0.80 showing extensive variation is an agreement with the results of Safavi *et al*., (2010) and Amini *et al.,* (2008) noticed in the range of 0.26 to 0.57 and 0.30 to 0.89, respectively. Similarly Pahani *et al.,* (2013) observed coefficient of similarities from 0.30 to 0.79.

In present research work, dendrogram using UPGMA cluster analysis divided all the 12 accessions into three main clusters and 4 sub-cluster. Similar cluster analysis based on complete method of Safavi *et al.,* (2010), 20 safflower accessions were separated into main 5 clusters with a cluster distance ranged from 0.27 to 0.85. Various others studies conducted by different investigators showed similar results such as Khan *et al.,* (2009), Mahasi *et al.,* (2009) and Pahani *et al.,* (2013).

Though using a small number of RAPD primers overall a considerable level of genetic diversity was observed by the experimental work conducted on twelve safflower germplasm. This study suggests that RAPD primers can be useful for the systematic study of this crop, parent plants selection for breeding programs and also can add to improve molecular propagation methods for the improvement of superior native safflower germplasm. This research will be quite helpful for the breeding improvement of safflower germplasm in the future.

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