Assessment of Molecular Variability of Safflower (Carthamus Tinctorius L.) Genotypes using ISSR Markers"

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ABSRACT: Safflower (*Carthamus tinctorius* L.), is a multi-purpose crop employed for diverse uses such as dye production, edible oil extraction and for medicinal applications. The unsaturated fatty acid of safflower lowers cholesterol. Most of the genetic diversity that local and traditional varieties possess are being lost currently. The DNA of 30 safflower germplasm accessions was subjected to PCR amplification using 15 ISSR primers. In the present study using ISSR primers a total 70 amplicons were found and in which 67 amplicons found polymorphic with an average polymorphism of 98 percent.On an average each primer produced 4.67 amplicons. The size of amplification product ranged from 100 bp to 1100 bp. ISSR primer P-825 produced maximum number of amplicons (8) .By using UPGMA cluster analysis the dendrogram depicting phylogenetic relationship among the selected genotypes. In the present investigation they were grouped into two major clusters that is A and B. The cluster A consists of 15 genotypes while cluster B consist of 15 genotypes. The cluster A was distinct from cluster B with 41% similarity. The PIC value generated by ISSR primer ranged from 0.0885 to 0.3725, with an average value of 0.2994. Highest PIC value recorded in primer P-829 (0.3725), whereas minimum PIC value recorded in primer NAGPUR SSR-8932799 (0.0885).

INTRODUCTION:

Safflower (*Carthamus tinctorius* L.), is the only cultivated species among 25 species of genus *Carthamus* having 2n=24 chromosomes (Ashri and Knowles, 1960). Safflower has been grown as commercial for its edible oil with very high levels of polyunsaturated fatty acids. Safflower seed contains 24-36% oil. The oil is as good as sunflower oil having enough amount of linoleic acid (78%), which is very useful for reducing blood cholesterol content. The unsaturated fatty acids of safflower lower this cholesterol. Most of the genetic diversity that local and traditional varieties possess is being lost currently. The new varieties are more genetically homogeneous and therefore more expose to pathogens and adverse environmentally conditions. The cake contains about 7.9% nitrogen, 1.9% potash and 2.2% phosphoric acid and its application as manure is supposed to greatly improve the physical properties of heavy soils.Safflower is cultivated in arid and semi-arid conditions in various agricultural zones across the world (Weiss 1983).

Various markers morphological, biochemical, and molecular are used to assess plant genetic diversity. With the advent of DNA markers, possessing the advantages of higher polymorphism and independent of environment and plant growth stage, they have been widely employed for the assessment of genetic diversity. Inter-simple sequence repeat (ISSR) is a DNA based marker with primers designed based upon dinucleotide, tetranucleotide or pentanucleotide repeats. ISSR markers, with the advantages of simplicity, acceptable stability and high reproducibility, have been successfully used in genetic variation studies, gene mapping, germplasm identification and fingerprinting construction. ISSR markers are more specific than RAPD markers, because of their longer SSR-based primers with higher primer annealing temperature, enabling amplifications of more reproducible bands. The ability to reveal genetic variation among different genotypes may be more directly related to the number of polymorphisms detected with each marker technique rather than a function of which technique is employed. Development of ISSR marker does not need prior knowledge of the genome to be analyzed; hence, it can be used universally for plant genome analysis. ISSR marker provides more polymorphism (Fang and Roose, 1997; Wolfe and Morgan, 1998) as compare to isozymes, because of the lack of mutational constants in the inter simple sequence repeats they are largely part of the non-coding regions of the genome, while isozymes are from the coding regions of the genome (Wolfe and Morgan, 1998).

REVIEW OF LITERATURE:

Sehgal and Raina (2005) studied genetic diversity in safflower using different markers like SSR, ISSR, AFLP and RAPD. Fourteen cultivars were used for the study. AFLP markers could fingerprint all the cultivars. Similarities between genotypes were calculated using Jaccard's coefficient and UPGMA clustering analysis.

Johnson *et al*. (2007) characterized safflower with Amplified Fragment Length Polymorphism (AFLP) markers to enhance germplasm management and utilization. AFLP analysis resulted in 102 unambiguous polymorphic primers. 96 accessions were taken for study.

Mahasi *et al*. (2009) conducted experiment to study genetic polymorphism in thirty-six Safflower accessions using Random amplified polymorphic DNA marker (RAPD). Statistical analysis was carried out using NTSYS and resemblance matrix was developed using UPGMA.

Derakhshan *et al*. (2014) evaluated and characterized forty two genotypes of six species of *carthamus* using EST-SSR marker.

Lee *et al*. (2014) carried out assessment of 100 safflower accessions using thirty microsatellite (SSR) markers.

Kumari *et al*. (2017) studied genetic diversity of 20 Safflower genotypes using morphological and Simple Sequence Repeat markers (SSR) markers. Total nine morphological traits like plant height, number of branches per plant, test weight, harvest index, seed yield and oil content showed significant variation. Among the tested SSR markers eleven primers showed amplification with seventeen polymorphic bands expressing 56% polymorphism.

Pavithra *et al*. (2017) estimated genetic variation of 118 genotypes using thirty eight SSR primers and screened twenty markers generated amplicons in 118 accessions.

Wodajo *et al*. (2015) observed that four selected ISSR primers produced a total of 43 bands across the 70 safflower accession. The number of amplified fragments with ISSR primers ranged from 6 to 15 per primer with varied in size of 100 to 1000 base pairs. The cluster analysis based on ISSR data safflower individual assembled from different localities and regions observed to be spread all over the trees without forming strict grouping based on their geographic origin. However, some individual from Amhara and Oromia tends to form separate groups. The majority of the groups observed in UPGMA and NJ trees were intermixed individuals from SNNPR and Tigray with Oromia and Amhara populations.

Ali *et al.* (2019) in 131 safflower accessions obtained from 28 countries were investigated using 12 ISSR markers. A sum of 201 ISSR bands were obtained among which 188 (93.844%) were found polymorphic. Mean polymorphism information content (0.448) and diversity parameters including mean effective number of alleles (1.655), mean Shannon's information index (0.557), mean expected heterozygosity (0.354) and mean overall gene diversity (0.377) showed a good level of genetic diversity in the studied safflower materials.

MATERIALS AND METHODS:

Table No. 1. List of safflower genotypes selected for the experiment:

Sr. No.	Genotypes	Sr. No.	Genotypes	Sr. No.	Genotypes
1.	SAF 1630	11.	GMU 2757	21.	SSF 12-40
2.	SAF 1672	12.	SSF 1507	22.	BHIMA
3.	SAFG 1853	13.	EC 302630	23.	SSF 748
4.	SAFG 1856	14.	SSF 1701	24.	SSF 658
5.	SAFG 1859	15.	SSF 1704	25.	SAF 1403
6.	SSF 1602	16.	SSF 1705	26.	SAF-P 1606
7.	SSF 1604	17.	SSF 1706	27.	SAF-P 1608
8.	SSF 1685	18.	GMU 4914	28.	NIRA
9.	SSF 1660	19.	SSF 15-48	29.	PBNS 12
10.	SSF 1889	20.	SSF 13-71	30.	TSF 1

Sr. No.	Primer ID	Sequence (5'-3')	Tm
1.	IS-12	GTGTGTGTGTGTGTTG	49°C

2.	P-334	AGAGAGAGAGAGAGAGYT	55°C
3.	P-825	ACACACACACACACACT	55°C
4.	P-827	ACACACACACACACACC	55°C
5.	P-829	ACACACACACACACACC	55°C
6.	P-841	GAGAGAGAGAGAGAGAYC	55°C
7.	P-868	ACACACACACACACACYG	55°C
8.	P-880	GGAGAGGAGAGGAGA	55°C
9.	UBC-840	GAGAGAGAGAGAGAGACT	55°C
10.	NAGPUR SSR-8932799	AGCAGCAGCAGCGG	55°C
11.	NAGPUR SSR-8932801	AGCAGCACGACGAT	55°C
12.	NAGPUR SSR-8932802	CACACACACACAAT	55°C
13.	NAGPUR SSR-8932806	CACACACACACAGA	55°C
14.	NAGPUR SSR-8932807	CACACACACACAAA	55°C
15.	ISSR-827	ACACACACACACACACG	55°C

Extraction of genomic DNA by C-TAB method. The qualitative analysis of genomic DNA was performed by agarose gel electrophoresis. Concentration of DNA was measured using UV visible Spectrophotometer (Nanodrop, ND-1000 USA) at 260 and 280 nm. After electrophoresis the band intensity of genomic DNA was visualized on gel documentation unit.

RESULT AND DISCUSSION:

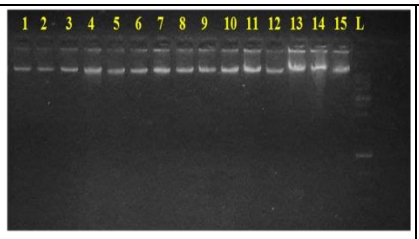
Table No.2 Polymorphic alleles generated by ISSR markers

Sr. No.	Primer Code	Size of Amplified product (~bp)	Total No. of amplicons	No. of polymorphic amplicons	No. of monomorphic amplicons	% Polymorphism	PIC
1.	IS-12	300-400	2	2	-	100	0.3515
2.	P-334	250-800	6	6	-	100	0.3228
3.	P-825	200-500	8	7	1	90	0.3702
4.	P-827	300-600	6	6	-	100	0.3566
5.	P-829	200-500	4	4	-	100	0.3725
6.	P-841	150-500	4	4	-	100	0.2673

7.	P-868	100-600	7	7	-	100	0.3353
8.	P-880	250-600	2	2	-	100	0.3582
9.	UBC-840	250-1000	6	6	-	100	0.2225
10.	NAGPUR SSR 8932799	500-800	2	2	-	100	0.0885
11.	NAGPUR SSR 8932801	300-600	4	4	-	100	0.1614
12.	NAGPUR SSR 8932802	300-600	4	4	-	100	0.3427
13.	NAGPUR SSR 8932806	250-900	7	7	-	100	0.2925
14.	NAGPUR SSR 8932807	350-1100	3	1	2	80	0.2776
15.	ISSR-827	200-550	5	5	-	100	0.3716
Total			**70**	**67**	**3**	**1470**	**4.4912**
Average			**4.67**	**4.47**	**0.2**	**98**	**0.2994**

Table No. 3. List of safflower genotype for cluster analysis with DNA based ISSR markers

Name of Cluster			**Name of safflower genotype**
Cluster A	A_1	A_{1a}	SAF-1630.
		A_{1b}	SAF-1672, SAFG-1856, SAFG-1859, SSF- 1604, SSF-1889, GMU-2757, SSF-1507, SSF-1704, EC-302630, SSF-1660, SSF-1701.
	A_2	A_{2a}	SAFG-1853, SSF-1685.
		A_{2b}	SSF-1602.
Cluster B	B_1	B_{1a}	SSF-1705.
		B_{1b}	SSF-1706.
	B_2	B_{2a}	GMU-4914, SAF-1403, SSF-1548, SSF-1371, SSF-1240, SSF-658, SSF-748, TSF-1, SAFP- 1606, SAFP-1608, NIRA, BHIMA.
		B_{2b}	PBNS-12.



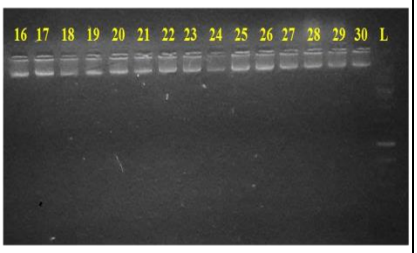


Plate No. 1. Genomic DNA of 30 safflower genotypes

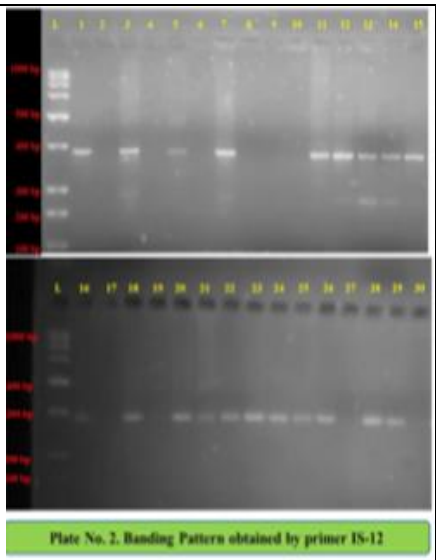
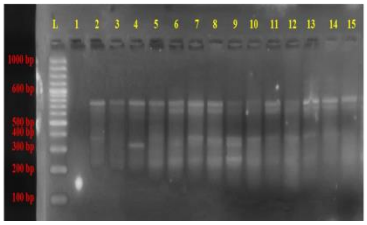


Plate No. 2. Banding Pattern obtained by primer IS-12



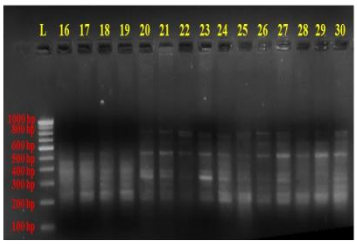
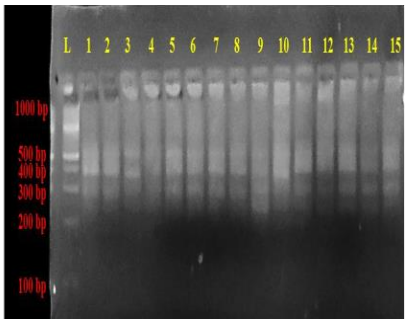


Plate No. 3. Banding Pattern obtained by primer P-334



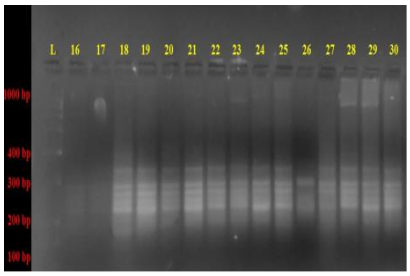
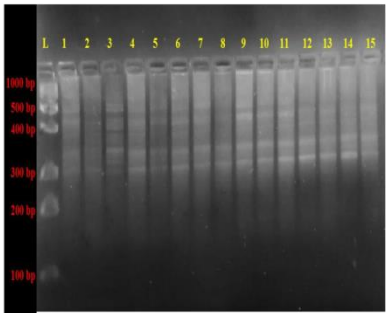


Plate No. 4. Banding Pattern obtained by primer P-825



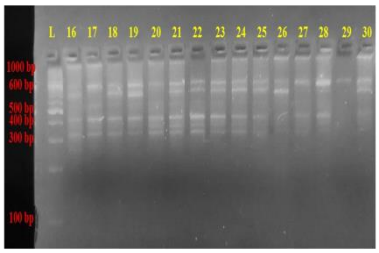
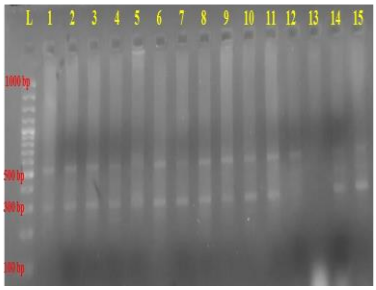


Plate No. 5. Banding Pattern obtained by primer P-827



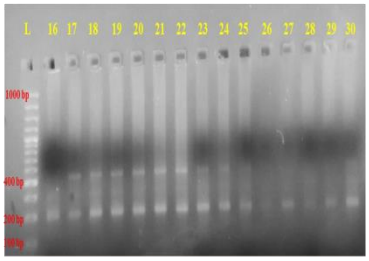
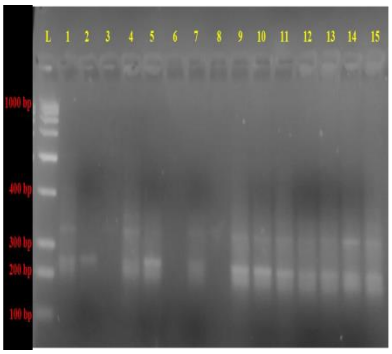


Plate No. 6. Banding Pattern obtained by primer P-829



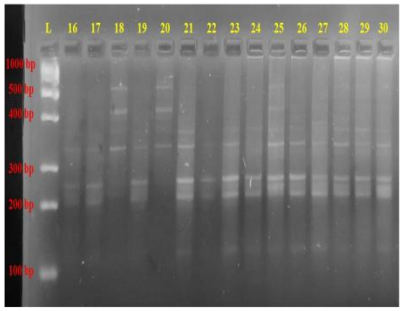
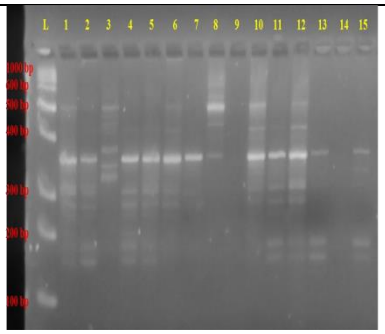


Plate No. 7. Banding Pattern obtained by primer P-841



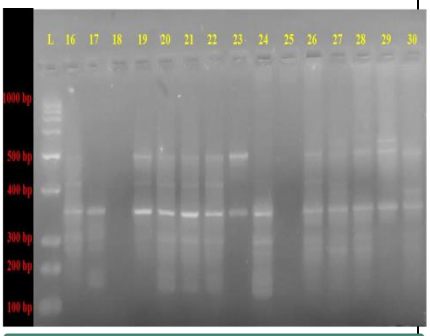
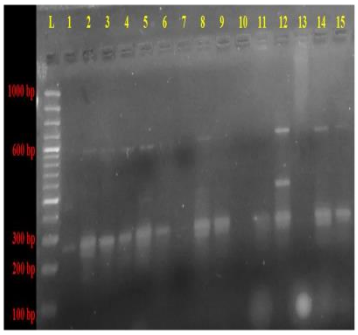


Plate No. 8. Banding Pattern obtained by primer P-868



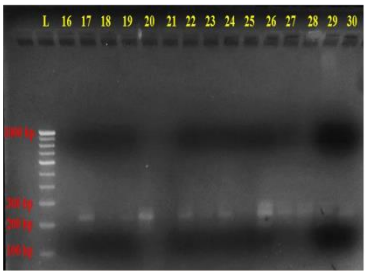
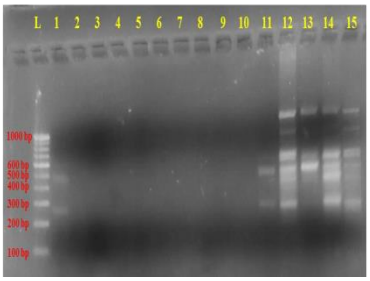


Plate No. 9. Banding Pattern obtained by primer P-880



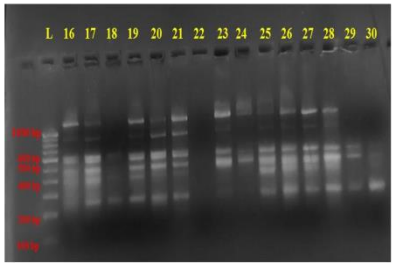
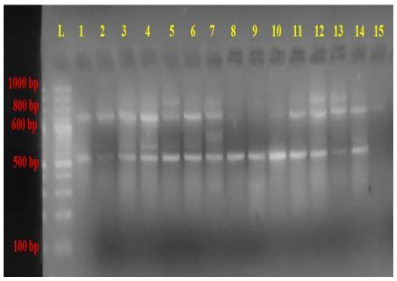


Plate No. 10. Banding Pattern obtained by primer UBC-840



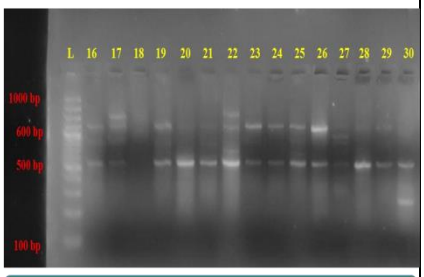
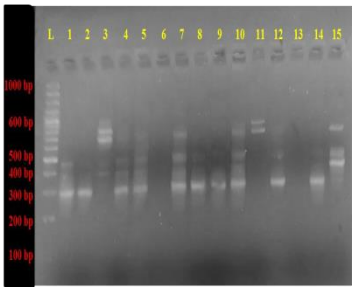


Plate No. 11. Banding Pattern obtained by primer NAGPUR SSR-8932799



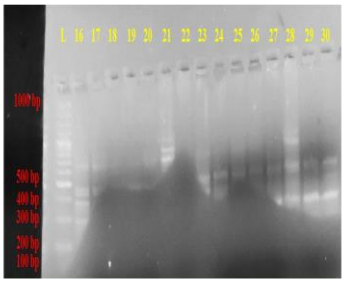


Plate No. 12. Banding Pattern obtained by primer NAGPUR SSR-8932801

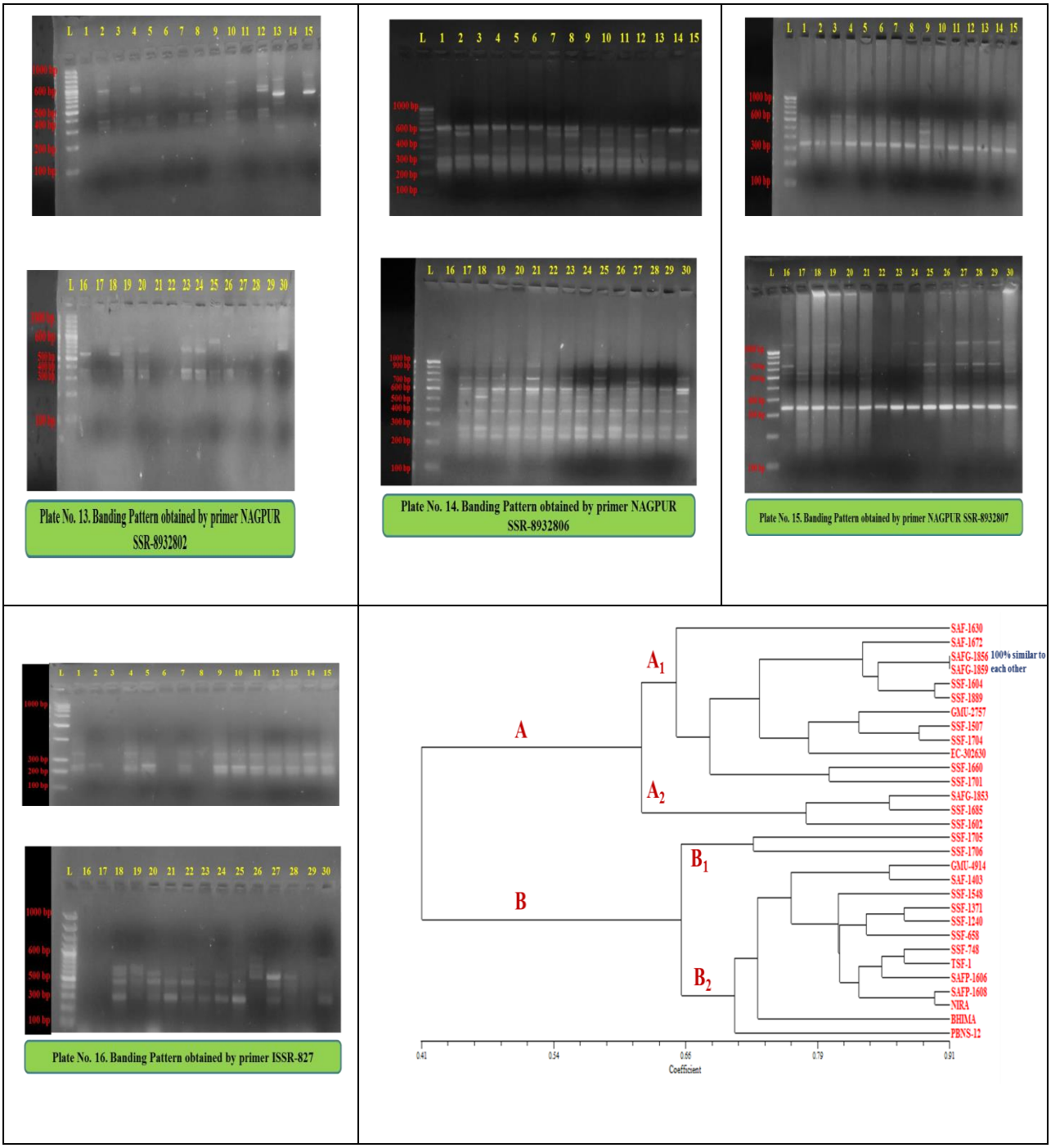


Plate No. 13. Banding Pattern obtained by primer NAGPUR SSR-8932802

Plate No. 14. Banding Pattern obtained by primer NAGPUR SSR-8932806

Plate No. 15. Banding Pattern obtained by primer NAGPUR SSR-8932807

Plate No. 16. Banding Pattern obtained by primer ISSR-827

Table N0.4. The Dice similarity coefficient value based on ISSR markers data

Genot	SAF	SAF	SAFG	SAFG	SAFG	SSF	SSF	SSF	SSF	SSF	GMU	SSF	EC	SSF	SSF	SSF	SSF	GMU	SSF	SSF	SSF	BHI	SSF-	SSF-	SAF	SAFP	SAFP	NIRA	PBN	TSF 1
SAF	1.0																													

1630	0																											
SAF1672	0.65	1.00																										
SAFG1853	0.48	0.65	1.00																									
SAFG1856	0.65	0.87	0.65	1.00																								
SAFG1859	0.70	0.81	0.70	**0.91**	1.00																							
SSF1602	0.58	0.67	0.72	0.68	0.67	1.00																						
SSF1604	0.62	0.81	0.71	0.82	0.84	0.65	1.00																					
SSF1685	0.52	0.77	0.85	0.74	0.71	0.82	0.74	1.00																				
SSF1660	0.65	0.71	0.55	0.75	0.74	0.70	0.71	0.67	1.00																			
SSF1889	0.62	0.82	0.67	0.87	0.84	0.70	0.90	0.78	0.77	1.00																		
GMU	0.70	0.74	0.57	0.75	0.78	0.58	0.75	0.61	0.64	0.78	1.00																	

2757																													
SSF1507	0.67	0.72	0.60	0.80	0.78	0.55	0.77	0.65	0.64	0.81	0.85	1.00																	
EC302630	0.60	0.61	0.44	0.64	0.64	0.54	0.68	0.50	0.57	0.67	0.75	0.75	1.00																
SSF1701	0.64	0.57	0.52	0.67	0.67	0.54	0.67	0.52	0.80	0.64	0.68	0.78	0.65	1.00															
SSF1704	0.67	0.70	0.54	0.77	0.75	0.52	0.74	0.60	0.65	0.72	0.80	0.88	0.82	0.80	1.00														
SSF1705	0.27	0.28	0.37	0.35	0.38	0.34	0.41	0.37	0.27	0.40	0.40	0.48	0.38	0.38	0.44	1.00													
SSF17006	0.48	0.44	0.37	0.51	0.50	0.48	0.42	0.44	0.45	0.47	0.51	0.58	0.48	0.51	0.57	0.72	1.00												
GMU4914	0.28	0.28	0.28	0.31	0.34	**0.21**	0.32	0.28	0.41	0.34	0.40	0.47	0.34	0.48	0.44	0.61	0.62	1.00											
SSF1548	0.38	0.40	0.30	0.47	0.44	0.32	0.37	0.38	0.37	0.47	0.48	0.58	0.47	0.42	0.55	0.67	0.78	0.77	1.00										
SSF1371	0.42	0.41	0.34	0.45	0.45	0.37	0.40	0.38	0.35	0.45	0.50	0.61	0.47	0.45	0.55	0.61	0.71	0.74	0.81	1.00									
SSF1	0.44	0.34	0.25	0.42	0.41	0.38	0.38	0.31	0.40	0.44	0.45	0.57	0.45	0.50	0.51	0.62	0.75	0.70	0.81	0.87	1.00								

240																													
BHIMA	0.41	0.42	0.32	0.50	0.48	0.51	0.42	0.40	0.48	0.48	0.40	0.47	0.40	0.40	0.41	0.51	0.65	0.61	0.71	0.77	0.87	1.00							
SSF748	0.35	0.37	0.34	0.38	0.35	0.30	0.44	0.41	0.34	0.44	0.44	0.55	0.44	0.42	0.52	0.64	0.65	0.75	0.81	0.80	0.80	0.71	1.00						
SSF658	0.38	0.41	0.28	0.42	0.42	0.28	0.42	0.32	0.32	0.42	0.48	0.54	0.45	0.38	0.51	0.67	0.70	0.75	0.80	0.84	0.82	0.77	0.87	1.00					
SAF1403	0.27	0.30	0.24	0.35	0.33	0.22	0.37	0.30	0.41	0.38	0.40	0.50	0.40	0.51	0.45	0.61	0.62	0.85	0.77	0.77	0.81	0.68	0.84	0.81	1.00				
SAFP1606	0.40	0.40	0.38	0.44	0.47	0.37	0.47	0.42	0.40	0.50	0.51	0.60	0.47	0.47	0.54	0.70	0.68	0.71	0.80	0.78	0.78	0.68	0.84	0.80	0.80	1.00			
SAFP1608	0.42	0.38	0.31	0.41	0.40	0.45	0.41	0.41	0.44	0.48	0.47	0.54	0.38	0.47	0.45	0.60	0.74	0.68	0.80	0.80	0.87	0.77	0.81	0.78	0.77	0.82	1.00		
NIRA	0.41	0.32	0.34	0.32	0.38	0.35	0.40	0.32	0.35	0.40	041	0.50	0.37	0.42	0.44	0.64	0.70	0.74	0.77	0.78	0.85	0.75	0.84	0.82	0.82	0.78	0.84	1.00	
PBNS12	0.35	0.30	0.45	0.32	0.35	0.37	0.38	0.47	0.31	0.41	0.44	0.47	0.37	0.34	0.41	0.64	0.64	0.64	0.67	0.70	0.65	0.60	0.80	0.74	0.67	0.77	0.72	0.74	1.00
TSF1	0.38	0.40	0.34	0.44	0.41	0.35	0.42	0.42	0.38	0.50	0.51	0.55	0.44	0.41	0.50	0.64	0.67	0.74	0.85	0.78	0.77	0.70	0.87	0.82	0.77	0.85	0.84	0.80	0.80	1.00

Thirty germplasm were screened for molecular characterization using ISSR markers. Among 25 ISSR primers used, 15 ISSR primers amplified genomic DNA. This produced 67 polymorphic amplicons with an average of 4.47 amplicons per primer. The number of amplicons generated by each primer varied from 8 amplicons (P-825) to 2 amplicons (IS-12, P-880, NAGPUR SSR-8932799.

The PIC value generated by ISSR primer ranged from 0.0885 to 0.3725. With highest PIC value observed in primer P-829 (0.3725) and lowest PIC value observed in primer NAGPUR SSR-8932799 (0.0885). The similarity coefficient between the genotypes varied from 0.41 to 0.91. The amplified product size ranged between 100 bp to 800 bp.

The UPGMA based cluster analysis using dice similarity coefficient grouped 30 safflower germplasm into two major clusters. The cluster A consists of 15 genotypes while cluster B consist of 15 genotypes. The cluster A distinct from cluster B with 41% similarity.The cluster A could be grouped into 2 sub clusters A_1 and A_2. The sub cluster A_1 consists of 12 genotypes, namely SAF-1630, SAF-1672, SAFG-1856, SAFG-1859, SSF-1604, SSF-1889, GMU-2757, SSF-1507, SSF-1704, EC-302630, SSF-1660, SSF-1701 while sub cluster A_2 consists of 3 genotypes, namely SSFG-1853, SSF-1685 and SSF-1602. The sub cluster A_1 distinct from sub cluster A_2 with 63% similarity. The cluster B could be grouped into 2 sub clusters B_1 and B_2. The sub cluster B_1 consists of 2 genotypes, namely SSF-1705, SSF-1706 while sub cluster B_2 consists of 13 genotypes, namely GMU-4914, SAF-1403, SSF-1548, SSF-1371, SSF-1240, SSF-658, SSF-748, TSF-1, SAFP-1606, SAFP-1608, NIRA, BHIMA and PBNS-12. The sub cluster B_1 distinct from sub cluster B_2 with 66% similarity.

Conclusions:

The results of molecular studies had demonstrated that there are differences in genetic diversity of genotypes. The ISSR markers have proved to be suitable for characterizing safflower germplasm.

Molecular characterization of 30 germplasm carried out using ISSR marker system shows clear differences among plant materials. The analysis showed that germplasm SSFG-1856 and SAFG- 1859 showed maximum similarity (100% similar to each other), whereas SAF-1630 and PBNS- 12 showed minimum similarity with each other.

Out of 15 ISSR primers 13 ISSR primers showed 100% polymorphism, namely IS-12, P-334, P- 827, P-829, P-841, P-868, P-880, UBC-840, NAGPUR SSR-8932799, NAGPUR SSR-8932801, NAGPUR SSR 8932802, NAGPUR SSR-8932806 and ISSR-827 and rest of two ISSR primers, namely P-825 and NAGPUR SSR-8932807 showed 90% and 80% polymorphism respectively.

It is therefore recommended that genetic conservation and improvement based on the selected materials should be encouraged and to identify diverse germplasm for developing varieties that suit the target environment. The screened material found superior need to be prioritized in terms of in-situ and ex-situ conservation for further evaluation and crop improvement.

Maximum similarity coefficient of (0.91) was found between genotypes SSFG-1856 and SAFG- 1859, whereas minimum similarity coefficient of (0.21) was observed between genotypes SSF- 1602 and GMU-4914.

The average size of amplification product ranged from 100 bp to 1100 bp.

The PIC value generated by ISSR primer ranged from 0.0885 to 0.3725. Highest PIC value observed in P-829 and lowest PIC value was observed in NAGPUR SSR-8932799.

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