Environment & Ecology 35 (1) : 1—6, January—March 2017
Website: environmentandecology.com ISSN 0970-0420

Genetic Diversity Revealed by SSR Markers in Safflower (*Carthamus tinctorius* L.) Germplasms

K. P. Pavithra, Rajesh S. Patil, Yallappa Harijan, B. R. Patil

Received 14 February 2016 ; Accepted 18 March 2016 ; Published online 6 April 2016

Abstract Safflower (*Carthamus tinctorius* L.) is mainly grown as oilseed crop in the arid and semiarid regions of the world. The present study was aimed at estimating genetic variation of 118 genotypes using 38 SSR primers and screened 20 markers generated amplicons in the 118 accessions. Out of 20 amplified markers, 8 markers showed polymorphism. A total of 48 amplicons (alleles) were scored for 20 SSR loci with a range of 1–8 alleles per locus with an average of 2.4 alleles per locus. The polymorphic information content value (PIC) calculated ranged from 0.00 to 0.66 with an average of 0.097 per marker. The safflower germplasms were divided into two main clusters each contained 5 sub clusters. Results of analysis of molecular variance showed a significant difference between germplasms.

Keywords Genetic diversity, Safflower, SSR markers, Cluster analysis, Germplasm.

Introduction

Safflower (*Carthamus tinctorius* L.) is a member of the composite, family Asteraceae. There are twenty five species in this genus. Out of which only *Carthamus tinctorius* L. (2n = 24) is cultivated. Safflower is cultivated mainly for its seeds, which yield edible oil traditionally. The crop was grown for its flowers, used for coloring and flavouring foods and making dyes, especially before cheaper aniline dyes became available, and in medicines. Besides, it contains 30% oil in India varieties. The oil constitutes 76% of Linoleic acid (PUFA) which helps in reducing cholesterol level in human blood.

Characterization of genetic variation in natural populations and breeding materials is crucial for effective conservation and exploitation of genetic resources for crop improvement programs. The analysis of DNA sequence variation is of great importance in genetic studies. In this context, molecular markers provide not only an effective tool to assess the genetic variation, but also a valuable genetic tool where conventional genetic studies have been difficult. The advantages of DNA markers are mainly due to the technical ease, low cost, high reproducibility, and availability of a number of marker systems that produce either dominant or codominant markers.

Microsatellites, known also as simple sequence repeats (SSRs), scattered throughout the genome are usually associated with a high level of frequency of length polymorphism [1]. SSR's offer some advantages over genomic DNA-based markers as they can be applied for analyses of functional diversity, more conserved and present in gene rich regions of the genome [2, 3] but they can still be used efficiently in assessing genetic relationships [4].

K. P. Pavithra, R. S. Patil, Y. Harijan*, B. R. Patil
Department of Genetics and Plant Breeding, University of Agricultural Sciences, Dharwad 580005, Karnataka, India
e-mail: pavithraak7060@gmail.com
e-mail: yallnh 4496@gmail.com
*Correspondence

Molecular markers have been used primarily for evaluation of germplasm assessment of the local cultivars and land races or germplasm accessions and to partition genetic variation geographically [5, 6, 7— 8, 9] and these will play a pivotal role in the management, characterization and utilization of germplasm. There was lack of congruence between agro-morphological and molecular matrices indicating the need for both measures for complete characterization of safflower diversity [6, 7—8, 9].

Currently, molecular approaches for safflower breeding are very limited. One of the most important components required for an efficient system for molecular breeding is the identification and characterization of suitable markers. Most molecular markers in safflower are randomly amplified nuclear markers i.e., random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSRs) and amplified fragment length polymorphism (AFLP). These are usually the markers of choice for crops with inadequate genomic resources. They have been used mainly for assessing genetic diversity in this species [5, 6]. In the present study we analyzed the all germplasm accessions of *Carthamus* by SSR markers with a view not only to assess their relative diversity but also to identify most suitable primers and markers for differentiating the germplasms.

Materials and Methods

Hundred and eighteen accessions of safflower (*Carthamus tinctorius* L.), including checks were selected for molecular diversity analysis (Table 1). 38 SSR primers were used for characterization of these genotypes.

DNA extraction

Two seeds of each of the selected accession were sown in small plastic pots. The genomic DNA from 10 days old seedlings was extracted from 3.0–5.0 g of leaf tissue using mini pre-rapid method with little modifications. The quality and concentration of the DNA was confirmed by electrophoresis on 0.8% agarose gel.

PCR amplification and electrophoresis

A set of 38 microsatellite DNA primers were used for the amplification of genomic DNA of all the 118 accessions. The SSR reaction mixture consisted of 25—50 ng of template DNA, 2 pM of Forward and Reverse primer, 2.5 mM of dNTPs, 1 unit of *Taq* polymerase (Bangalore Genei, India), 10 × PCR buffer (100 mM Tris pH 9.0, 500 mM KCL, 15 mM MgCl2 and 0.1% Gelatin) in a volume of 10 µl. Eppendorf thermal cycler gradient was used for cyclic amplification of DNA (Thermocycler). PCR amplification was carried out using Master Thermal Cycler 5331 - Eppendorf version 2.30, 31–09, Germany. The initial denaturation was at 94°C for 5 minutes followed by 38 cycles of 30 seconds at 94°C, 45 seconds at 56°C, 45 seconds at 72°C and final 10 minutes extension at 72°C. The electrophoresis of PCR amplified products was carried out on agarose gel of 2.5% in 1 × TAE at 120V for 3 hours. 5µl of tracking dye was added to 10 µl of PCR products and mixed well before loading into the wells. The electronic images of ethidium bromide (0.5 µg/ml) stained gels were captured and documented using Uvidoc (model: UVIDOC DOC - 008-XD).

Data scoring and statistical analysis of diversity

The products of PCR were scored visually by comparing with the standard marker of size 100 bp (Bangalore Genie). The amplicons obtained with regard to the expected size were scored by denoting ‘0’ (for absent) and ‘1’ (for present), each of which was treated as an independent character regardless of its intensity, thus producing a binary data matrix. Data from the SSR studies was analyzed using various statistical programs. Allelic variation was calculated from the frequencies of genotypes at each locus as the polymorphic information content (PIC). Genetic parameters namely major allele frequency, genotype frequency and polymorphic information content (PIC) were estimated using the software program Power Marker version 3.25 [10]. Dendrogram was constructed using the neighbourhood–joining algorithm using the program DARwin 5.0 [11] and the per cent polymorphism was calculated by using the following formula

Table 1. List of accessions and check varieties used in molecular characterization of safflower (*Carthamus tinctorius* L.).

Sl. No.	Accessions	Sl. No.	Accessions	Sl. No.	Accessions	Sl. No.	Accessions
1	HUS-305	31	GMU 3658	61	GMU 3708	91	GMU 3757
2	GMU 3625	32	GMU 3659	62	GMU 3711	92	GMU 3759
3	GMU 3626	33	GMU 3661	63	GMU 3716	93	GMU 3760
4	GMU 3628	34	GMU 3662	64	GMU 3719	94	GMU 3761
5	GMU 3631	35	GMU 3663	65	GMU 3720	95	GMU 3762
6	A-1	36	GMU 3664	66	GMU 3721	96	GMU 3764
7	GMU 3632	37	GMU 3666	67	GMU 3722	97	GMU 3768
8	GMU 3633	38	GMU 3667	68	GMU 3723	98	GMU 3769
9	GMU 3634	39	GMU 3668	69	GMU 3726	99	GMU 3770
10	GMU 3635	40	GMU 3671	70	GMU 3727	100	GMU 3771
11	GMU 3636	41	GMU 3672	71	GMU 3729	101	GMU 3772
12	GMU 3637	42	GMU 3673	72	GMU 3730	102	GMU 3774
13	MANJIRA	43	GMU 3674	73	GMU 3731	103	GMU 3775
14	GMU 3638	44	GMU 3676	74	GMU 3733	104	GMU 3780
15	GMU 3639	45	GMU 3677	75	GMU 3734	105	GMU 3781
16	GMU 3642	46	GMU 3678	76	GMU 3736	106	GMU 3782
17	JSF-1	47	GMU 3681	77	GMU 3737	107	GMU 3783
18	GMU 3643	48	GMU 3682	78	GMU 3738	108	GMU 3786
19	GMU 3644	49	GMU 3684	79	GMU 3739	109	GMU 3789
20	GMU 3645	50	GMU 3686	80	GMU 3740	110	GMU 3790
21	GMU 3646	51	GMU 3687	81	GMU 3741	111	GMU 3791
22	GMU 3647	52	GMU 3690	82	GMU 3743	112	GMU 3792
23	GMU 3648	53	GMU 3691	83	GMU 3744	113	GMU 3793
24	GMU 3649	54	GMU 3693	84	GMU 3746	114	GMU 3794
25	BHIMA	55	GMU 3694	85	GMU 3748	115	GMU 3797
26	GMU 3650	56	GMU 3699	86	GMU 3749	116	GMU 3798
27	GMU 3651	57	GMU 3700	87	GMU 3750	117	A-2
28	GMU 3652	58	GMU 3704	88	GMU 3753	118	NARI
29	GMU 3653	59	GMU 3705	89	GMU 3754		
30	GMU 3656	60	GMU 3706	90	GMU 3756		

$$\text{Per cent polymorphism} = \frac{\text{Number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

Results and Discussion

DNA based markers hold greater promise with several advantages viz., high polymorphism, insensitive to environment, stability and independent of developmental stages of plants. Characterization of genetic variation in natural populations and breeding materials is crucial for effective conservation and exploitation of genetic resources for crop improvement programs. The analysis of DNA sequence variation is of great importance in genetic studies. In this context, molecular markers are not only an effective tool to assess the genetic variation, but also help in the reinforcement of conventional genetic studies.

In the present study the results revealed that out of 38 SSR primers screened 20 markers generated amplicons in the 118 accessions (Table 2). Out of 20 amplified markers, 8 markers showed polymorphism. A total of 48 amplicons (alleles) were scored for 20 SSR loci with a range of 1–8 alleles per locus with an average of 2.4 alleles per locus. The size of the bands ranged from 162 bp to 397 bp. Majority of the markers amplified a single allele per marker. The polymorphic information content value (PIC) calculated ranged from 0.00 to 0.66 (acetolactate synthase–dr) with an average PIC value of 0.097 per marker. Similar PIC value range, allele number and allelic frequency in safflower were reported by many researchers with various markers [6, 12, 13].

To understand the relationships among the accessions, genetic dissimilarity matrix was estimated

Table 2. Number of alleles, allele frequency, gene diversity and polymorphic information content of 118 safflower (*C. tinctorius* L.) accessions for molecular characterization.

Sl. No.	Marker	Allele (bp)	Major allele frequency	Sample size	Number of observations	Allele number	Gene diversity	PIC
1	ACC oxidase 2-drought stressed	182	0.480	118	100	5	0.548	0.445
2	Acetolactate synthase–dr	341	0.459	118	111	8	0.705	0.667
3	Solute carrier family 7	397	0.955	118	89	3	0.087	0.084
4	G protein-coupled receptor	344	0.972	118	108	3	0.054	0.054
5	MYB133	386	0.810	118	42	5	0.329	0.308
6	Carbonic anhydrase 3	381	1.000	118	109	1	0.000	0.000
7	Gamma-TMT	368	0.957	118	93	3	0.083	0.081
8	GB-CT-081 micro-satellite	202	1.000	118	108	1	0.000	0.000
9	GB-CT-035 micro-satellite	298	0.912	118	114	4	0.165	0.160
10	GB-CT-011 micro-satellite	380	0.922	118	103	5	0.147	0.144
11	GB-CT-057 micro-satellite	300	1.000	118	106	1	0.000	0.000
12	GB-CT-023 micro-satellite	312	1.000	118	112	1	0.000	0.000
13	GB-CT-006 micro-satellite	383	1.000	118	113	1	0.000	0.000
14	GB-CT-094 micro-satellite	223	1.000	118	105	1	0.000	0.000
15	NADH dehydrogenase subunit 3	278	1.000	118	112	1	0.000	0.000
16	CT31ECT3 micro-satellite	215	1.000	118	116	1	0.000	0.000
17	Stearoyl-acyl carrier protein desaturase	162	1.000	118	118	1	0.000	0.000
18	CFFM28356	212	1.000	118	117	1	0.000	0.000
19	CFF(LMS) safflower	259	1.000	118	116	1	0.000	0.000
20	Aquaporin	356	1.000	118	98	1	0.000	0.000
	Mean		**0.923**		**105**	**2**	**0.106**	**0.097**

through unweighted pair group method with arithmetic mean (UPGMA) using DARwin 5.0 software. The results of hierarchial clustering in this study grouped the accessions into two main clusters, 'a' and 'b' and these were divided into ten sub-clusters. Among the two main clusters, Cluster 'a' formed 5 sub clusters in which cluster 2 had more number of accessions (19) followed by Cluster 1, 4, 3 and 5 with the number of accessions 17, 16, 11 and 3, respectively. Main Cluster 'b' had 5 sub clusters and among them Cluster 6 had more number of accessions (16). Among the accessions greatest genetic distance was found between accession GMU 3681 and GMU 3748 (Figure 1 and Table 3). Genetic diversity of safflower germplasm by using various markers were reported by many researchers [5, 6, 12, 14—17].

However, the cluster analysis in the present study indicated wide range of variability at genotypic level. The polymorphism at genotypic level can be assessed by generating high polymorphic markers. High resolution of markers is needed for characterization and evaluation which can be done by increasing the number of repeat units and length of the marker.

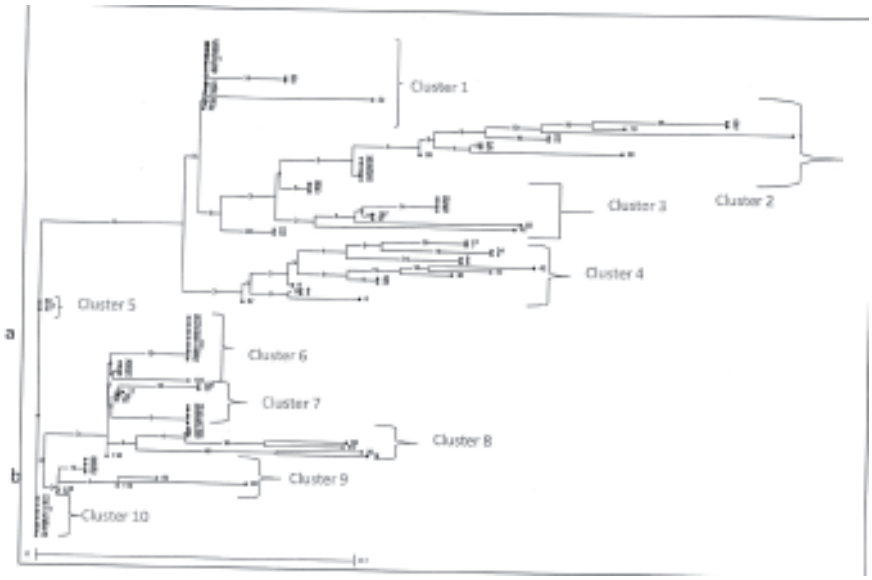

Fig. 1. Neighbor joining phenogram of 118 accessions based on genotypic data using DARwin 5.0.

Conclusion

Diversity analysis in the crops reveals greater variability present in the accessions for variable traits. In the present study the accessions taken for molecular diversity analysis has done conventional diversity also. So the comparision in this result is interesting because, the accessions GMU 3625, GMU 3626,

Table 3. Cluster distribution of 118 safflower (*C. tinctorius* L.) accessions including checks based on molecular characterization.

Cluster No.	Number of accessions	Name of the accessions
1	17	GMU 3681, GMU 3652, GMU 3682, GMU 3686, GMU 3794, GMU 3711, GMU 3653, GMU 3684, GMU 3694, GMU 3658, GMU 3690, GMU 3761, GMU 3650, GMU 3659, GMU 3656, GMU 3694, GMU 3757
2	19	HUS-305, GMU 3637, Manjira, GMU 3635, GMU 3667, GMU 3638, GMU 3639, GMU 3664, GMU 3666, GMU 3668, GMU 3706, GMU 3700, GMU 3756, GMU 3693, GMU 3699, GMU 3738, GMU 3768, GMU 3769, GMU 3737
3	10	GMU 3741, GMU 3691, GMU 3783, GMU 3651, GMU 3674, GMU 3676, GMU 3677, GMU 3727, GMU 3744, GMU 3672
4	15	GMU 3632, GMU 3636, GMU 3662, GMU 3625, GMU 3631, A-1, GMU 3643, GMU 3671, GMU 3678, GMU 3663, GMU 3633, GMU 3628, GMU 3626, GMU 3634, GMU 3722
5	3	GMU 3723, GMU 3793, GMU 3708
6	16	GMU 3705, GMU 3649, GMU 3733, GMU 3734, GMU 3754, GMU 3762, GMU 3797, NARI-6, GMU 3770, GMU 3764, GMU 3759, GMU 3739, GMU 3705, GMU 3760, GMU 3731, GMU 3775
7	11	GMU 3771, GMU 3642, JSF-1, GMU 3643, GMU 3720, GMU 3780, GMU 3774, GMU 3781, GMU 3772, GMU 3782
8	8	GMU 3789, GMU 3790, GMU 3786, GMU 3719, GMU 3721, GMU 3644, GMU 3645
9	9	GMU 3746, GMU 3749, GMU 3753, GMU 3716, GMU 3743, GMU 3792, GMU 3740, GMU 3798, GMU 3736
10	10	GMU 3646, GMU 3647, GMU 3648, GMU 3730, A-2, Bhima, GMU 3726, GMU 3750, GMU 3729, GMU 3748

GMU 3684 and GMU 3760 fell in solitary clusters when both morphological and molecular based clustering was done. It supports conventional results of variability parameters.

References

1. Kalia RK, Rai MK, Kalia S, Singh R, Dhawan AK (2011) Microsatellite markers: An overview of the recent progress in plants. Euphytica 177 : 309—334.
2. Zhang LY, Bernard M, Leroy P, Feuillet C, Sourdille P (2005) High transferability of bread wheat EST-derived SSRs to other cereals. Theor Appl Genet 111 : 677—687.
3. Guo W, Wang W, Zhou B, Zhang T (2006) Cross-species transferability of G. arboreum-derived EST-SSRs in the diploid species of *Gossypium*. Theor Appl Genet 112 : 1573—1581.
4. Gupta PK, Rustgi S (2004) Molecular markers from the transcribed / expressed region of the genome in higher plants. Funct Integr Genomics 4 : 139—162.
5. Sehgal D, Raina SN (2006) Genotyping safflower (*Carthamus tinctorius*) cultivars by DNA fingerprints. Euphytica 146 : 67—76.
6. Johnson RC, Kisha TJ, Evans MA (2007) Characterization safflower germplasm with AFLP molecular markers. Crop Sci 47 : 1728—1736.
7. Yang YX, Wu W, Zheng YL, Chen L, Liu RJ, Huang CY (2007) Genetic diversity and relationships among safflower (*Carthamus tinctorius* L.) analyzed by inter-simple sequence repeats (ISSRs). Genet Resour Crop Evol 54 : 1043—1051.
8. Amini F, Saeidi G, Arzani A (2008) Study of genetic diversity in safflower genotypes using agro-morphological traits and RAPD markers. Euphytica 163 : 21—30.
9. Khan MA, Witzke-Ehbrecht SV, Brigitte L, Heiko C, Becker (2008) Relationships among different geographical groups, agro-morphology, fatty acid composition and RAPD marker diversity in Safflower (*Carthamus tinctorius*). Genet Resour Crop Evol 56 : 19—30.
10. Liu K, Muse SV (2005) Power Marker: Integrated analysis environment for genetic marker data. Bioinformatics 21 : 2128—2129.
11. Perrier X, Flori A, Bonnot F (2003) Data analysis methods, Sci Publ, Montpellier, pp 43—76.
12. Golkar P, Arzani A, Rezaei AM (2011) Genetic variation in safflower [*Carthamus tinctorious* (L.)] for seed quality-related traits and Inter-Simple Sequence Repeat (ISSR) Markers. Int J Mol Sci 12 : 2664—2677.
13. Hamdan YAS, Garcia-moreno MJ, Redondo-nevado J, Velasco L, Pe rez-vich B (2011) Development and characterization of genomic microsatellite markers in safflower [*Carthamus tinctorius* (L.)]. Pl Breeding 130 : 237—241.
14. Ravikumar RL, Soregaon CD, Satish D (2007) Molecular diversity analysis of five different species of genus *Carthamus*. Nat Sem on Changing Global Vegetable Oils Scenario: Issues and Challenges before India, 29—31 Jan. DOR, Hyderabad, pp 2—4.
15. Chapman MA, Hvala J, Strever J, Matvienko M, Kozik A, Michelmore RW (2009) Development, polymorphism, and cross-taxon utility of EST–SSR. Theor Appl Genet 120 : 85—91.
16. Mayelhofer R, Archibald C, Bowles V, Good Ag (2010) Development of molecular markers and linkage maps for the *Carthamus* species *Carthamus tinctorius* and *Carthamus oxyanthus.* Genome 53 : 266—276.
17. Talebi M, Mokhtari M, Rahimmalek M, Sahhafie SR (2012) Molecular characterization of *Carthamus tinctorius* and *C. oxyacanthus* germplasm using sequence related amplified polymorphism (SRAP) markers. Pl Omics J 5 : 136—142.