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The Linkage Map of *Carthamus tinctorius* L. Based on RAPD, SRAP and EST-SSR Markers

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ABSTRACT

Carthamus tinctorius L. (2n = 2x = 24), commonly known as safflower, is widely cultivated. The genetic map of safflower (*C. tinctorius*) constructed using RAPD, SRAP and EST-SSR markers. An F2 population of 117 progenies derived from a cross between cultivated *C. tinctorius* L. and a wild accession from *C. oxyacanthus* species was used to generate the map during 2015-2016. A total of 101 markers, including 49 RAPD, 40 SRAP and 22 EST-SSR were identified and used for genetic linkage group analysis. The linkage map consisted of 9 major linkage groups. The map covered a total length of 1760.9 cM with an average marker density of 31.4 cM between two adjacent markers. In the safflower map, 56 markers (41 RAPD, 14 SRAP and 1 EST-SSR) were linked. Construction of linkage map is an important first step towards detecting genes controlling agronomically important traits and can show the location of genetic loci along the chromosomes.

Keywords: Genetic map, Safflower, Accession, Molecular markers, Genetic control.

INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is an ancient crop with numerous past and present uses. The leaves, petals, and seeds have tremendous medicinal and therapeutic significance, and petals are also used for extracting dye for coloring cloths and food stuffs (Danisova *et al*., 2020; Golparvar *et al*., 2016).

Genetic linkage mapping dates back to the early 20th century when scientists began understanding the recombinational nature and cellular behaviour of chromosomes. Sturtevant (1913) studied the first genetic linkage map of chromosome X of *Drosophila melanogaster* and now molecular linkage maps have been constructed for most of the plant species (Saliba-colombani *et al*. 2010). The advent of arbitrarily primed PCR-based assays has been made possible the efficient detection of DNA polymorphisms and the generation of large numbers of markers for fingerprinting and for genetic mapping of qualitative and quantitative traits (Crespel *et al*., 2002).

In this study, our initial objective was to construct an interspecific genetic linkage map of *C. tinctorius* using the F_2 population based on random amplified polymorphic DNA (RAPD), expressed sequence tag- simple sequence repeat (EST-SSR) and sequence-related amplified polymorphism (SRAP) molecular markers. Molecular tagging and mapping information for quality traits and disease and pest resistance is expected to be useful for safflower breeders because of the possibility of using molecular markers for marker-assisted selection in their breeding programs.

MATERIALS AND METHODS

Plant material and DNA isolation

An F_2 family was produced by the controlled interspecific hybridization between one female *C. tinctorius* L. genotype C_{111} (spineless and white seeded, and red in flower colour) and one male *C. oxyacanthus* species, genotype ISF_2. (Spiny, black seeded and yellow in flower colour). The male parent was a wild-stock collected previously from Isfahan Province, Iran and the female parent was a cultivated-stock (pure line) selected from land race Kooseh. Female parent was emasculated with some modifications and after that was pollinated with male parent. F_1 hybrids were inter-crossed to produce the F_2 population during 2015-2016. Seeds obtained from the cross-pollination were germinated in a lightly moistened potting mix at room temperature. Healthy seedlings were transplanted into a greenhouse one month after seed germination. Leave samples from a total of 117 F_2 progenies and two parents that were in 4 leaves stage were collected and stored at −20 ˚C for bulked genomic DNA extraction (Murray and Thompson 1998). The DNA was quantified electrophoresis using lambda standard DNA on 0.8% agarose gels.

RAPD analysis

A set of 91 oligonucleotide random primers, each 10 nucleotides long, obtained from Operon Technologies were used on F_2 plants for PCR reaction. The PCR reaction was performed in a 25 µl volume. The reaction mixture contained: 2.5 µl 10× PCR buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 20 ng genomic DNA, 0.8 µM primer and one U of *Taq* DNA polymerase (Biotherm, Germany). Amplification was performed in 0.2 ml tubes placed in a Techgen PCR thermal cycler (Biometra, Germany) under the following temperature program: 1 cycle of 5 min at 94 °C (denaturation), 1 min at 36 °C (annealing), and 1 min at 72 °C

(extension); followed by 40 cycles of 1 min at 94 °C, 1 min at 36 °C, and 1 min at 72 °C ending with 1 cycle of 5 min at 72 °C (final extension). PCR amplified products were separated by electrophoresis on 1.2% agarose gels using Biometra Model S2 gel electrophoresis equipped with Biometra (Model PS9009TC) power supply at 50 W for 2 h in 1× TBE buffer. Gels were stained with ethidium bromide (0.5 mg/ ml). DNA banding patterns were visualized using Biometra Gel Documentation (Model P755874X02).

SRAP analysis

A total of 11 different primer combinations were employed for the SRAP analysis (Table 1). PCR reaction solution was prepared in a total volume of 25 µl that contained 2.5 µl of 10 × PCR buffer, 2.5 µl of $MgCl_2$ (25 mM/l), one U of *Taq* DNA polymerase (Dingguo), 0.5 µl of dNTP (10 mM/l), one µl of primer (10 µM/l), and 20 ng of template genomic DNA. Amplification was carried out in a Peltier Thermal Cycler PTC-0200 (Biorad Co) with the following PCR program: 94ºC for 5 min followed by 5 cycles of 94ºC for 60 s, 35ºC for 60 s, and 72ºC for 60 s; and 35 cycles of 94ºC for 60 s, 50ºC for 60 s, 72ºC for 60 s; and a final extension at 72ºC for 10 min. The PCR products were mixed with 10 ml of formamide loading buffer (95% formamide, 20 mM EDTA, pH 8.0, Xylene cyanol and Bromophenol blue) analyzed on 8% non-denatured polyacrylamide gels in 1x TBE buffer running at 200 V constant voltage for 2.5 h and then silver stained according to the reported procedure (Bassam *et al*., 1991; Liu *et al*., 2007).

Table 1. SRAP primers sequences used in the study

Forward	**Reverse**
Me1:TGAGTCCAAACCGGATA	Em1:GACTGCGTACGAATTAAT
Me2:TGAGTCCAAACCGGAGC	Em2:GACTGCGTACGAATTTGC
Me3:TGAGTCCAAACCGGAAT	Em3:GACTGCGTACGAATTGAC
Me4:TGAGTCCAAACCGGACC	Em4:GACTGCGTACGAATTTG
Me5:TGAGTCCAAACCGGAA	Em5:GACTGCGTACGAATTAAC
	Em6:GACTGCGTACGAATTGCA

Table 2. Selected primer sequences used for RAPD analysis

Primer	**Sequence**	**No.of markers**	**Primer**	**Sequence**	**No.of markers**
CGS-15	GGACACCACT	3	CGS-40	GGGTGTGGTT	5
CGS-16	CCGATATCCC	2	CGS-41	CAACCCACAC	6
CGS-18	GGTAGCAGTC	6	CGS-50	CCTCTAGACC	10
CGS-20	CAGTTCGAGG	3	OPA-01	CAGGCCCTTC	3
CGS-25	CAGTTCTGGC	6	OPC-13	AAGCCTCGTC	3
CGS-26	AGCCAGCGAA	6	OPA-04	AATCGGGGCTG	7
CGS-27	AATCGGGCTG	6	OPH-04	GGAAGTCGCC	7
CGS-37	CCACCCACAC	5	OPK-07	AGCGAGCAAG	3

EST-SSR analysis

SSR markers were selected from two sources (Chapman *et al*., 2007; Chapman *et al*. 2009). Out of 66 markers, 14 microsatellite primer pairs were selected that showed high polymorphism in the parents ' population. Polymerase chain reaction (PCR) amplification was performed in a total volume of 15µl containing 1×PCR buffer, 0.2 mM dNTPs, 0.4 µM of each forward and reverse primers, 1.5 mM $MgCl_2$, one U of *Taq* polymerase (Fermenthas) and 20 ng of total genomic DNA. All reactions were performed using an Eppendorf thermal cycler (Germany). Touch-down PCR program was used as follows: 94°C for 5 min followed by 10 cycles of 94°C for 1 min, 68°C for 1 min, and 72°C for 1 min; and 35 cycles of 94°C for 5 min; 58°C for 1 min, and 72°C for 1 min, and final extension was allowed to proceed at 72°C for 10 min. Amplified products were separated on 8% denaturing polyacrylamide gel electrophoresis in 1×TBE buffer along with 100 bp DNA ladder (Fermenthas) as size marker. PCR products were visualized by silver staining (Bassam *et al*., 1991).

Statistical analysis

RAPD, SRAP and EST-SSR primers were assayed first on four random progenies and both parents. Primers showing polymorphism between parents and segregating in the progenies were selected for subsequent use in the whole mapping population. Markers were scored in the mapping population of 117 F_2 individuals for their presence or absence in two different data sets according to their parental origin. All markers were tested for a Mendelian segregation to compare observed and expected ratios of 1:2:1 in the F2 population (for EST-SSR analysis) or 3:1 (for SRAP and RAPD analyses) using Chi-square analysis ($P < 0.05$). Those markers segregating in a Mendelian fashion were used for linkage map construction. Markers were scored as A, B, C and D for RAPD and SRAP analysis and A, B and H for EST-SSR markers. Linkage relationship among markers was derived with the software package MAPMAKER/EXP v. 3.0 (Lander *et al*., 1987) and then genetic linkage map was constructed. Linked and unlinked groups were first determined with Mapmaker software with the "group" command. "Compare" command was then applied to determine the most appropriate markers with the maximum distance (50 cM) and the most Likelihood of odds (LOD) value (LOD > 3). Linkage distances were calculated for each linked group with the "map" command using Kosambi's mapping function. Data obtained were finally used to construct a chromosomal map of linkage groups using Draw map software (Liu and Merg, 2003).

RESULTS AND DISCUSSION

Marker segregation

Among the 91 RAPD primers, 16 primers (17.5%) (Table 2) showed reproducible and clearly scorable polymorphism between the parents (C111-ISF$_2$). These markers generated 81 polymorphic dominant loci. Each RAPD primer produced two to ten polymorphic DNA markers with an average of 5.06 RAPD loci per primer. Primers with higher GC content produced higher number of RAPD bands, as has been reported for other plant species (Fritsch *et al.*, 1993). Among all RAPD markers, 32 (39.5%) showed a distorted segregation (Table 3).

Screening out of 11 combinations of the SRAP primers, 16 revealed highly polymorphic patterns and were chosen for genetic mapping with the whole population. These 16 primer pairs generated a total of 87 markers with an average of 5.43 markers per primer pair. Out of 87 SRAP markers, 35(40.2%) displayed distorted segregation (Table 4).

Table 3. Chi-square test for segregation distortion of RAPD markers in F2 population

Markers	Genotype A/A or A/B	B/B or A/B	X^2	Direction of distortion	Markers	Genotype A/A or A/B	B/B or A/B	X^2	Direction of distortion
CGS-25-4	63	44	14.41**	ISF2	OPA-01-3	53	55	36.42**	ISF2
CGS-27-2	53	58	41.9**	C111	CGS-27-6	53	58	42.01**	ISF2
CGS-27-1	63	47	17.75**	C111	CGS-40-5	51	59	45.64**	C111
CGS-18-1	45	56	45.25**	C111	CGS-26-2	60	46	18.36**	ISF2
CGs-37-5	58	54	31.02**	ISF2	OPA-01-1	52	54	35.50**	ISF2
OPH-04-6	63	48	18.99**	ISF2	OPK-07-2	61	46	17.74**	ISF2
OPH-04-2	51	61	49.85**	C111	CGS-18-3	46	57	46.18**	C111
OPH-04-3	69	40	7.9**	ISF2	CGS-50-2	53	55	36.42**	C111
CGS-37-1	60	46	18.2**	ISF2	CGS-50-6	48	60	50.32**	C111
OPA-04-4	47	55	41.7**	C111	CGS-50-7	52	56	39.02**	C111
CGS-41-4	51	65	58.9**	ISF2	CGS-50-1	62	46	17**	C111
CGS-41-1	68	48	16.45**	ISF2	OPH-04-3	63	48	28.9**	ISF2
OPC-13-3	45	62	57.4**	C111	CGS-25-2	41	64	66.18**	C111
CGS-40-2	59	51	25.58**	ISF2	CGS-25-1	57	50	25.4**	C111
CGS-50-8	53	55	36.42**	C111	OPA-04-2	49	54	38.05**	ISf2
CGS-91-5	51	65	59.08**	ISF2	CGS-18-4	53	50	28.48**	ISF2

** Significant at the 0.05 probability level.

Table 4. Chi-square test for segregation distortion of SRAP markers in F2 population

Markers	Genotype A/A or A/B	B/B or A/B	X^2	Direction of distortion	Markers	Genotype A/A or A/B	B/B or A/B	X^2	Direction of distortion
M4E2-2	35	47	42.4**	ISF2	M4E2-3	51	29	55.5**	C111
M4E2-4	35	47	20.05**	C111	M4E3-1	57	43	49.14**	C111
M3E3-6	47	43	33.59**	C111	M5E2-1	62	38	64.48**	C111
M5E2-2	54	46	40.8**	C111	M5E2-3	48	51	34.17**	ISF2
M5E2-4	49	51	33.28**	ISF2	M5E2-5	45	55	20.7**	C111
M5E2-6	61	38	10.76**	ISF2	M1E3-1	68	47	15.21**	ISF2
M1E3-2	64	50	57.52**	C111	M1E3-3	78	37	4.13**	ISF2
M1E3-5	59	55	42.47**	C111	M1E3-7	74	40	6.1**	ISF2
M1E3-8	71	43	9.65**	ISF2	M1E4-1	45	69	12.48**	C111
M1E4-5	46	68	14.03**	C111	M1E5-1	50	63	21.7**	C111
M1E5-2	76	39	101.8**	C111	M3E1-1	17	82	152.17**	ISF2
M3E1-2	58	40	14.03**	ISF2	M3E1-3	48	50	32.72**	ISF2
M3E1-5	47	51	26.16**	C111	M2E6-4	43	70	10.05**	C111
M5E4-1	33	66	80.33**	ISF2	M5E4-2	58	37	57.6**	C111
M5E4-3	50	47	27.01**	ISF2	M5E4-4	50	47	33.64**	C111
M2E2-1	60	41	57.12**	C111	M4E4-1	67	35	80.43**	C111
M4E4-2	60	42	14.32**	ISF2	M4E4-3	37	64	8.47**	C111
M4E5-1	57	45	47.14**	C111	M3E6-1	42	69	9.55**	C111
M3E6-2	61	50	50.7**	C111	M3E6-3	73	38	5.08**	ISF2
M2E6-3	18	96	5.02**	C111	M2E6-4	43	71	9.65**	C111
M2E5-3	60	53	28.05**	ISF2	M2E5-4	50	62	22.27**	C111
M2E5-5	65	43	12.35**	ISF2					

** Significant at the 0.05 probability level.

Genetic linkage maps

An average of 77(39.4%) of the markers (39.5%, 40.2%, and 18% of the RAPD, SRAP and EST-SSR markers, respectively) deviated significantly ($P < 0.05$) from the expected 3:1 and 1:2:1 ratios between the homozygous genotypes. Skewed segregation has been reported primarily on populations derived from interspecific and inter-generic crosses.

Of the 101 DNA markers screened in F2 population, 56 (55.4%) were mapped and 45 remained unlinked. Nine linkage groups were formed as opposed to the expected twelve based on the haploid chromosome number of the species (Figure 1). The linkage groups varied widely in length, from 27.5 to 959.4cM (Table 5). Three linkage groups (LG1, LG2 and LG3), were longer than 100 cM, whereas 6 linkage groups (LG4 to LG9), were less than 100 cM but longer than 25 cM. The map covered a total length of 1760.9 cM with an average marker density of 31.4 cM between two adjacent markers. The maximum number of markers was found in the linkage groups LG1 and LG2, respectively. Among the 56 markers placed on the genetic linkage map of *C. tinctorius* (Figure 1), 24 are distributed on LG1, 13 on LG2,4 on LG3, 3 on LG4, LG5 and LG6 and 2 on LG7, LG8 and LG9 (Table 6).

In the present study, we created a F_2 mapping population to construct genetic maps of C. *tinctorius* and *C. oxyacanthus*. These species belong to *Carthamus* section, and have a significant divergence in geographical distribution and important morphological traits, such as shapes of leaves and colors of flower and seeds. However, a significant divergence between the parents may inhibit exchange and recombination between chromosomes, which may contribute to the low rate of the linkage re-organization, and cause the serious segregation distortion phenomenon, reducing the credibility of linkage maps and the applicable scope.

However; Wild species of safflower, *C. oxyacanthus* is highly crossable with cultivated species, *C. tinctorius* L. and could be directly exploited in broadening the safflower gene pool and improving the crop for biotic and abiotic stress environments with negligible effect on the oil quality of cultivated safflower (Sabzalian *et al*., 2008).

The most common DNA fingerprint technologies differ substantially in (i) the complexity of technological procedures, (ii) the required amount of DNA, (iii) sequence information needed for a genome being scanned, (iv) the analytical power of assigning genotype relatedness, (v) expense in technology consisting labor and money, and (vi) broadness of applications. In this context, RAPD and SRAP fingerprinting seem to have wide potential for applications in molecular ecology, and requires the least in technology and labour expenses and template genomic DNA. Another advantage of these techniques is the high level of polymorphism in many plant species. Especially, these types of DNA markers can be detected without any prior knowledge of the genome sequence. Due to its advantages of highly polymorphic production, easy manipulation, reliability, moderate throughput and easy sequencing of selected bands, these genotyping approaches have been successfully applied in several species for different purposes (Ferriol *et al*., 2003; Budak *et al*., 2004; Dawei *et al*. 2010; Gao *et al*., 2019).

In our study, of the 91 RAPD primers tested, 16 primers (17.5%) produced 81 polymorphic bands. The degree of RAPD polymorphism was agreed with bread wheat (Suenaga *et al*., 2015) in terms of polymorphic RAPD primers. Polymorphic band per RAPD primers were found to be 5.06.

Table 5. linkage groups identified based on RAPD, SRAP and EST-SSR markers

Linkage group	No. of Markers	Length(cM)	Avg distance (cM)
LG1	24	959.4	39.9
LG2	13	381.5	29.3
LG3	4	118.8	29.7
LG4	3	63.6	21.2
LG5	3	27.5	9.1
LG6	3	84.8	28.2
LG7	2	33.4	16.7
LG8	2	44.8	22.4
LG9	2	47.1	23.5
Total	56	1760.9	220

Table 6. Number of markers mapped on each linkage group

Markers	Linkage groups								
	LG1	LG2	LG3	LG4	LG5	LG6	LG7	LG8	LG9
RAPD	19	11	4	2	2		1	2	
SRAP	4	2		1	1	3	1		2
ESR-SSR	1								

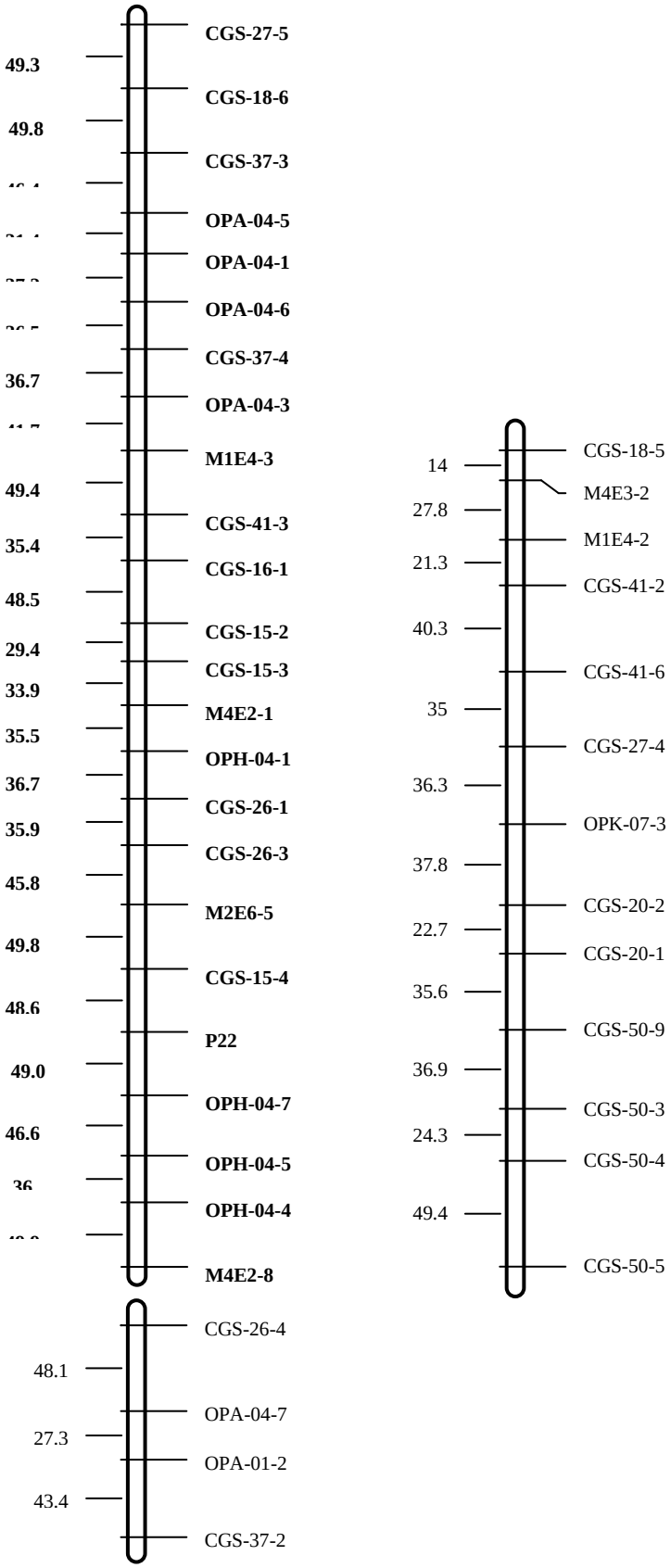
In the current study, the SRAP method produced nearly the same polymorphic markers per primer (5.43 markers per primer pair) as RAPD (5.06 markers per primer) while in *Dendrobium* it was 9.8 markers per primer pair in SRAP and 3.8 markers per primer in RAPD (Dawei *et al*., 2010). SRAP primers were also more polymorphic between parents (53.3%) than RAPD primers (17.5%) although sequencing gel electrophoresis is required to resolve SRAP markers.

Out of 66 tested EST-SSR markers, 14(21.21%) showed polymorphism between the parent populations. The most markers used were mapped alone and just one marker was linked on one linkage group. The reason for such a low number of marker linkages in linkage group may be attributed to even distribution of EST-SSR markers in the genome.

Segregation distortion is a quite common phenomenon in the plant kingdom and has been reported in many species. In tetraploid cotton, Zhang *et al*. (2016) detected markers showing distorted segregation. A variety of genetic factors could result in segregation distortion, including pollen tube competition, pollen lethal, preferential fertilization, and selective elimination. On the other hand, in F_2 population dominant homozygote and heterozygote could not be distinguished clearly and therefore it may lead to segregation distortion too.

Higher LOD threshold could improve the quality of map, but it causes fewer markers useful for map construction and lower genome coverage for the constructed linkages. In this study, we used a LOD threshold of 3.0. The *C. tinctorius* map had 9 major linkage groups. These linkage group numbers developed here are lower than the haploid chromosome number of the *Carthamus* (n = 12). The 9 major linkage groups possess regions with large genetic distances (9.4 to 49.9 cM) between markers [figure 1], indicating that they might be within chromosomal regions with frequent recombination events.

The map covered a total length of 1760.9 cM with an average marker density of 31.4 cM between two adjacent markers while intra-specific map of Mayerhofer *et al*. (2010) covered a total genome length of 896 cM. They mapped 190 AFLP and SSR markers on 12 linkage groups of two inter and intra-specific maps. In the present study, only 14 SRAP and 1 EST-SSR markers have been mapped and this will cause low coverage of the genome. The study to be continued, other types of DNA markers, such as AFLP and SSR, may be used for a saturated mapping of *Carthamus* species. In addition, more powerful marker systems like single nucleotide polymorphism (SNP) might have been remained to be generated for safflower.


CGS-27-5
49.3
CGS-18-6
49.8
CGS-37-3
OPA-04-5
OPA-04-1
OPA-04-6
CGS-37-4
36.7
OPA-04-3
M1E4-3
49.4
CGS-41-3
35.4
CGS-16-1
48.5
CGS-15-2
29.4
CGS-15-3
33.9
M4E2-1
35.5
OPH-04-1
36.7
CGS-26-1
35.9
CGS-26-3
45.8
M2E6-5
49.8
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OPH-04-7
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OPH-04-4
M4E2-8
CGS-26-4
48.1
OPA-04-7
27.3
OPA-01-2
43.4
CGS-37-2
CGS-18-5
14
M4E3-2
27.8
M1E4-2
21.3
CGS-41-2
40.3
CGS-41-6
35
CGS-27-4
36.3
OPK-07-3
37.8
CGS-20-2
22.7
CGS-20-1
35.6
CGS-50-9
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CGS-50-5
LG1
LG2
LG3

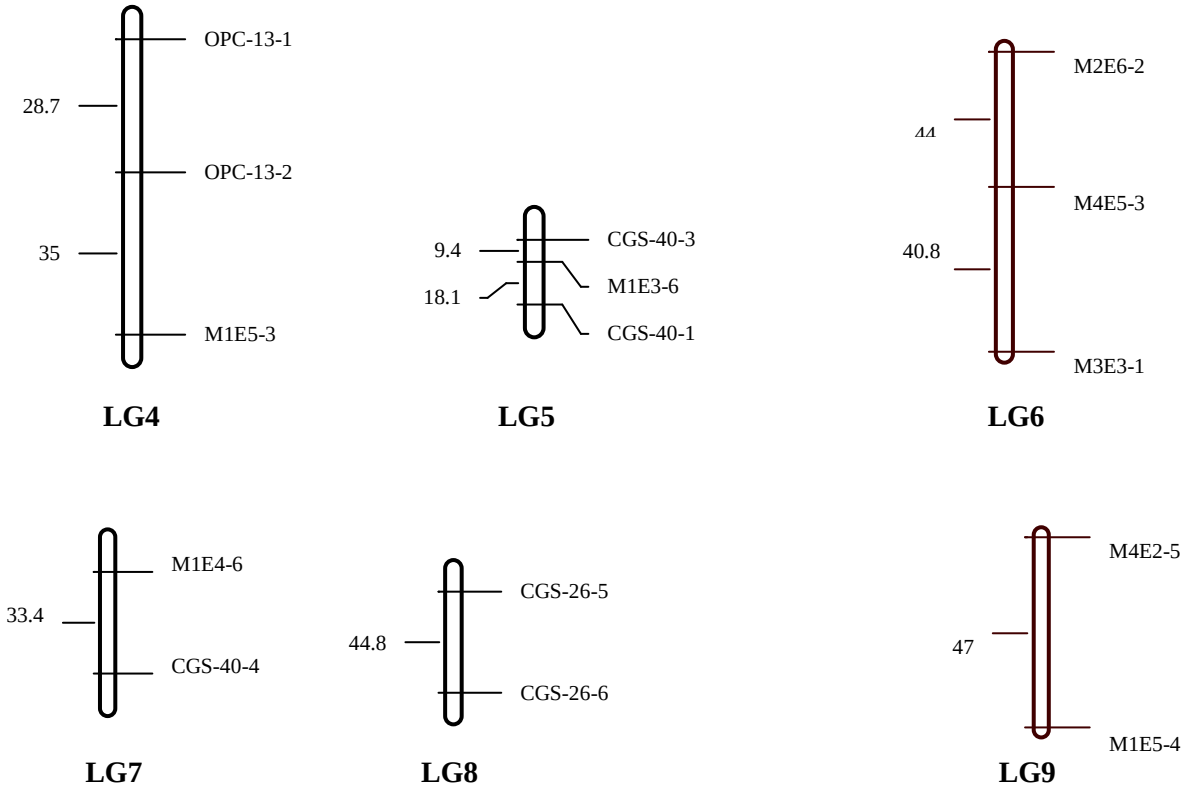

Figure 1. Genetic linkage map of *Carthamus* consisting of RAPD, SRAP and SSR markers. Distances in cM are indicated to the left and marker name to the right of each linkage group.

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