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Original scientific article

THE GENETIC DIVERSITY OF SAFFLOWER (*Carthamus tinctorius* L.) GENOTYPES DEVELOPED BY HYBRIDIZATION USING SSR MARKERS

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Safflower is an annual oilseed crop which has healthy edible oil containing high amount of unsaturated fatty acids in the world. In this study, we investigated safflower registered cultivars of Turkey (4), genotypes retrieved from USDA (10) and their hybrids (45) for genetic variation using 10 simple sequence repeat (SSR) loci. Genetic diversity calculated registered cultivars, genotypes and hybrids were as follows: mean number of alleles (4.67), expected heterozygosity (0.680), average effective number of alleles (3.172), and polymorphism information content (0.664). The dendrogram analysis revealed at least four possible major clusters in the parents and hybrids. High level of genetic diversity explained between the populations and Fst calculate (0.593) suggested that the clusters were differentiated to each other. Registered safflower cultivars of Turkey were distributed across all four clusters and the accessions from USA were defined in most of the clusters. The dendrogram based method analysis revealed two major clusters which corresponded to spiny and spineless safflower genotypes. It was suggested that the studied 6 SSR markers could be utilized for safflower breeding studies based on molecular analysis.

Key words: Carthamus tinctorious L., cultivars, genetic diversity, oilseed, oil content

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INTRODUCTION

Safflower (*Carthamus tinctorius* L., *Asteraceae*) is an annual oilseed crop that is adapted to hot and dry environments. It is one of the oldest multipurpose oilseed crops in the world. Traditionally, safflower is grown for its valuable oil seeds, flowers and used as fabric dye, food colouring and for medicinal purposes (EMONGOR, 2010; CULPAN and ARSLAN, 2022). The cultivated safflower varieties are diploid and have 24 chromosomes (GARNATJE *et al*., 2006). Safflower is a significant oilseed crop because of its worldwide economic importance in oilseed industry. In the recent years, the importance of safflower has not only increased as oilseed crop, but also in the production of biodiesel (DORDAS and SIOULAS, 2008).

The total production of safflower seed was 590.869 t in the world in 2019. The highest production was recorded in Kazakhstan with 199.789 t, followed by 88.130 t in USA, 81.189 t in Russia, 51.655 t in Mexico and 24.640 t in India. Also, the production of safflower seed was 21.883 t and yield were 1379 kg ha^{-1} in Turkey (FAO, 2019).

Genetic diversity in the safflower germplasm collection and hybrids were investigated to achieve these goals through targeted breeding purposes using molecular markers (JOHNSON *et al*., 2007; YANG *et al*., 2007; KHAN *et al*., 2009; SEHGAL *et al*., 2009; YAMINI *et al*., 2013; LEE *et al*., 2014; DERAKHSHAN *et al*., 2014; PEARL and BURKE, 2014; KUMAR *et al*., 2015; AMBREEN *et al*., 2015; USHA KIRAN *et al*., 2015). Microsatellites or SSRs are considered to be the most suitable for assessment of genetic diversity. Simple sequence repeat (SSR) markers consist of repeats of simple nucleotide motifs, which are abundant in most eukaryotic genomes. They have gained importance because of their high degree of polymorphism, co-dominant, multi-allelic, reproducibility and locus specificity (PHILIPS and VASIL, 2001; BRAKE *et al*., 2022). Many SSR markers have been developed for safflower in recent years (MAYERHOFER *et al*., 2010; YAMINI *et al*., 2013; LEE *et al*., 2014; AMBREEN *et al*., 2015). However, the use of SSR markers in the analysis of genetic diversity in large collections of safflower core accessions and recently developed varieties has not yet been reported.

The present study was conducted to assess genetic diversity in 45 safflower genotypes developed by hybridization and their 14 parents using SSR markers.

MATERIALS AND METHODS

Plant materials

A total of 59 safflower (*Carthamus tinctorius* L.) genotypes (14 parents and 45 hybrids (Hybrid BA1-BA24; Hybrid EC1-EC21)) were examined to determine the genetic variation using SSR markers in Tekirdağ Namık Kemal University, Faculty of Agriculture, Department of Field Crops (Turkey). Two of the four registered safflower varieties were obtained from the Transitional Zone Agricultural Research Institute (Dinçer and Balcı), two were obtained from the Trakya Agricultural Research Institute (Linas and Olas), while ten genotypes were obtained from the United States Department of Agriculture (USDA). Especially genotypes obtained from USDA were selected for their important breeding objectives such as seed yield and oil content. The varieties and genotypes were hybridized and 45 safflower F_1 hybrids were obtained. Table 1

shows the characteristics of the registered varieties and genotypes used as parent material in this study. Hybridizations and molecular analyzes were carried out in the fields and laboratories of the Faculty of Agriculture, University of Tekirdağ Namık Kemal.

Table 1. The characteristics of the registered varieties and selected genotypes

No/ Cultivar	Accession	Flower Color and Spininess	Oil Content (%)	Seed Yield (kg da^{-1})	Oleic Acid Content (%)	Linoleic Acid Content (%)
1	PI 193473	O, Sp	34.35	157.58	14.09	69.83
3	PI 209287	Y, Sp	33.41	104.88	20.58	62.37
10	PI 253520	Y, Sp	29.82	105.58	29.75	55.59
21	PI 369842	O, Sl	34.58	161.24	18.36	63.03
25	PI 506427	O/Y, Sl	31.35	40.14	19.90	61.72
28	PI 537601	Y, Sl	32.00	144.99	21.89	61.06
29	PI 560161	Y, Sp	35.98	52.19	17.55	65.80
30	PI 560177	Y/R, Sp	34.68	71.19	30.79	51.67
31	PI 572432	Y/R, Sp	36.04	160.09	22.81	57.04
35	PI 603208	S, Sp	37.42	84.19	62.36	24.24
Dinçer	-	O/R, Sl	26.52	164.20	13.99	74.54
Balcı	-	Y, Sp	33.06	136.77	13.55	75.98
Linas	-	Y, Sp	34.16	197.57	17.69	71.51
Olas	-	Y, Sp	32.80	192.57	74.12	16.88

O: Orange, Y: Yellow, R: Red, Sp: Spiny, Sl: Spineless

Genomic DNA isolation and PCR amplification

Total genomic DNA was isolated from young fresh leaves (25-30 old days of seedling, 10-15 cm in length) of safflowers from each genotype, variety and hybrid using a modified Cetyl Trimethyl Ammonium Bromide (CTAB) method (DOYLE and DOYLE, 1990). The seeds of the plant material were planted in the field and germinated. The fresh safflowers leaves were crushed in porcelain mortars, and DNA extraction was carried out using manual CTAB method. The quality and amount of DNA was identified using NanoDrop ND-1000 spectrometer (NanoDrop Technologies, Wilmington, DE, USA) and total DNA was diluted to a working concentration of 25 ng/µl. Also, the final diluted DNA samples were run on 1% agarose gel using electrophoresis to determine the DNA quality.

Sequences of SSR primers were chosen after referring to the published literature. Gradient PCR was performed to determine the annealing temperature of each SSR primers. The SSR primer sequences and melting temperatures are shown in Table 2. DNA was amplified by PCR in a total volume of PCR reaction volume was 15 µl, including of 50 ng genomic DNA, 200 µM of each dNTP, 0.5 µM primer (Medsantek), 1X Taq DNA polymerase buffer (100 mM Tris-HCL, pH: 8.3, 2 Mm $MgCl_2$ and 0.01% gelatin), and 1.25 unit Taq DNA polymerase enzyme (Thermo Scientific, USA). The PCR was performed in a thermal cycler (Applied Biosystems,

CA, USA) according to the PCR protocol of 5 min at 95 °C for one cycle; 45 sec at 95 °C, 30 sec at annealing temperature of each SSR marker and 72°C for 1 min for 35 cycles, and finally 7 min at 72 °C (AMBREEN *et al*., 2015). The PCR products were checked on 1% agarose gel stained with RedSafe (Nucleic Acid Staining Solution, iNtRON Biotechnology) using horizontal electrophoresis. The gels were visualized by the Vilbert Lourmat UV imaging system.

The PCR products were run in a 6% denaturing polyacrylamide gel including acrylamide:bis-acrylamide (19:1) and TBE 1X. A 6 µl sample of each PCR product was mixed with 2 µl of gel loading buffer (Thermo Scientific, USA) and heated at 95°C for 5 min for denaturation and immediately placed in ice until loaded. Electrophoresis was then conducted at 15 W for 4.5 hour in a vertical electrophoresis (Kodak, New York, USA). The gel was stained with silver dye, and the bands were defined.

Table 2. The selected SSR marker's specific primers sequences (forward and reverse), and the annealing temperature used in PCR amplification

SSR Marker	Primer Sequences (5ˈ – 3ˈ)	Annealing Temp. (°C)	References*
NGSaf-69	F: TCTCATCAACGATAAAGCAGAATC	62	a
NGSaf-69	R: TCAACTTCATCTTTTCACGATTTC		
NGSaf-83	F: GCCAAACCCTAACACAGAATCA	62	a
NGSaf-83	R: CGGTTGTGCCCTAGCTTTTA		
NGSaf-94	F: CCATCGAAACTCTACAAAACCC	60	a
NGSaf-94	R: AGGACAAAAGAGGGAATGATGA		
NGSaf-130	F: TGCGACTTGTGTTTCTTCTTCCC	62	a
NGSaf-130	R: AAAAGCCGTCCGGTGAAATTG		
NGSaf-142	F: GATGTTAACCTGTGTACCATCTGC	60	a
NGSaf-142	R: CGTCTAATGAACACTCAATCCAAA		
NGSaf-145	F: GAGCATGAAACGGAGAATTAGG	60	a
NGSaf-145	R: TCAACAGTAGCAGATCCTTCCA		
NGSaf-154	F: TGATATCAATGGTATGATTTTCCTTT	62	a
NGSaf-154	R: GGATGGCGAACAAGATTACAA		
NGSaf-238	F: AACAGTGGGCCTGATATGTTTT	62	a
NGSaf-238	R: CGGCTAATCCAAACCCTAGAAT		
SSR-5	F: TGATGTTCTTGATGAGTCAATGC	63	b
SSR-5	R: CATGTTAGCAAGCATTTGTGG		
SES-85	F: GGGTTCACTTCTTTCTCTCTC	63-64	b
SES-85	R: AGTACTCCTCCAGTGACATACAG		

*The reference for each SSR marker is indicated with "a" for AMBREEN *et al*., (2015), "b" for KUMARI *et al.,* (2017)

Data analysis

The PCR product allele sizes for each SSR loci were calculated with GelAnalyzer (version 19.1) by the 100 bp DNA ladder as a standard. The SSR alleles were checked by two different persons to provide accuracy. The polymorphism information content (PIC), which estimates the allelic diversity, was calculated using the formula $PIC = 1-\sum pi^2$, where p_i is the

frequency of i^{th} allele of the SSR locus (NEI, 1975). The power of discrimination (PD) was calculated using the formula $PD = 1-\sum gj^2$, where g_j is the frequency of the j^{th} genotype of the SSR locus. The PD gives an estimation of the probability for two individuals to exhibit different allele profiles for the same locus. Jaccard's (1908) similarity coefficients (or Jaccard's index) were calculated, recorded in a matrix, and used to design a dendrogram with an online program (Dendro UPGMA) using the unweighted pair-group method with arithmetic average (UPGMA) cluster analysis. Effective number of alleles, Shannon's information index, observed heterozygosity, expected heterozygosity and average heterozygosity were calculated using POPGENE (Version 1.32) (YEH *et al*., 1997).

RESULTS AND DISCUSSION

Fifty-nine safflower genotypes, varieties and hybrids were DNA fingerprinted in the current study using six of the 10 SSR markers (NGSaf-69, NGSaf-94, NGSaf-130, NGSaf-145, NGSaf-238 and SES-85). PCR products of SSR markers were checked using 0.7% agarose gel (Figure 1a). The good PCR bands of all safflower with all SSR primers were defined and their ranges of the band sizes matched to the ranges revealed in literature (AMBREEN *et al*., 2015; KUMARI *et al*., 2017). PCR products were run the in 6% denaturing polyacrylamide gel, which is a useful method for an evaluation as it permits the discrimination to the 1 bp resolutions for SSR analysis. Six of the markers were polymorphic, two were monomorphic (NGSaf-142 and NGSaf-154) and two markers (NGSaf-83 and SSR-5) could not amplify after PCR optimization in studied genotypes. While NGSaf-142 marker had 454 bp allele ranges, NGSaf-154 marker had 500 bp allele ranges in our study.

MAYERHOFER *et al*. (2010), HAMDAN *et al*. (2011), YAMINI *et al*. (2013) and LEE *et al*. (2014) have identified several polymorphic SSR markers for safflower breeding. For this study, 10 SSR markers were carried out based on literature information that defined as polymorphic SSR markers in previous studies. USHA KIRAN *et al*. (2015) revealed that SSR allelic diversity in the core subset of safflower germplasm was low (NA = 3.6, He = 0.314 and PIC = 0.284). In their study only three out of 44 markers (ct-47, ct-50 and ct-246) had high PIC values (>0.5), which may be used for diversity applications in safflower. While, HAMDAN *et al*. (2011) announced an average allele number as 3.2 per SSR locus among ten safflower cultivars, BARATI and ARZANI (2012) revealed that EST-SSR alleles had average of 3.43 per locus in a collection of 48 genotypes.

Table 3 presents SSR marker names, observed product sizes, number of alleles, size of alleles, PIC, DP, MI and HE of the 6 primers analyzed in the current study. When parental and hybrids were evaluated, SSR markers with two and more than two alleles were evaluated as polymorphic loci. When 6 polymorphic SSR markers were examined, a total of 28 alleles were detected (Table 3). Average polymorphic information content (PIC) was calculated as 0.664 among parent and crossbreeds of safflower genotypes. The highest number of alleles was NGSaf-69 and NGSaf-238 markers with the 6 alleles among the 6 SSR markers that determined as polymorphic. The average number of alleles was 4.67 that include all markers. In the current study, the allele sizes varied from 208 bp for SES-85 to 408 bp for NGSaf-94 (Table 3). The allele size variations in a marker were proportional to the sequences repeat length for four of the

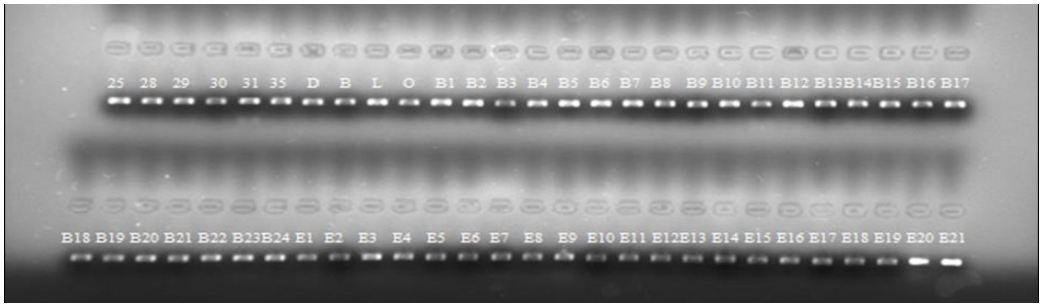
six SSR markers studied. Average effective number of alleles (Ne) 3.172, average observed heterozygosity (Ho) 0.548, average expected heterozygosity (He) 0.680, mean coefficient of selfing (Fit) 0.187, average genetic variance (Fst) 0.593 and the average Shannon information index (I) was 1.251 in the studied genotypes. The highest effective allele number was determined at the NGSaf-69 marker (0.413) and the lowest effective allele number at the NGSaf-145 marker (2.516). The observed heterozygosity varied between 0.288 (NGSaf-130) and 0.915 (NGSaf-69); and the expected heterozygosity varied between 0.608 (NGSaf-145) and 0.786 (NGSaf-69). While, the NGSaf-130 marker had the highest selfing coefficient and total genetic variance (0.560 and 0.780, respectively), the NGSaf-69 marker was the lowest (-0.174 and 0.413). The polymorphic information content (PIC) value varied among the markers between 0.6395 (NGSaf-145) and 0.6778 (NGSaf-238 and SES-85), while the Shannon information index value was determined between 0.993 (NGSaf-145) and 1.618 (NGSaf-69) values. USHA KIRAN *et al*. (2015) reported the PIC values ranged from 0.04 to 0.695 with an average of 0.322 in safflower germplasm. Similar with to this study, an average of 2.8 alleles per SSR locus, He = 0.386 and PIC = 0.325 in a 100 safflower accessions were revealed by LEE *et al*. (2014). According to DERAKHSHAN *et al*. (2014), it has a low SSR polymorphism (NA = 3.81 and PIC = 0.30) was found in a collection that contained a few accessions of wild species. FILIPPI *et al*. (2015) also revealed an average of NA = 4.95, He = 0.51 and PIC = 0.50 in a collection of 170 sunflower accessions using 42 SSR markers. RAVI (2014) revealed that the average observed heterozygosity was 0.088 and the average expected heterozygosity was 0.332. Also, he observed the average PIC value was 0.286. USHA KIRAN *et al*. (2015) observed low Ho (0.002) which caused by high inbreeding due to a lack of outcrossing during maintenance of the germplasm collection, whereas LEE *et al*. (2014) reported higher Ho (0.452). In our study, Ho was calculated as a 0.548 similar with LEE *et al*. (2014). RAVI (2014) observed very low Ho, suggesting the possibility that only a negligible amount of out-crossing occurred between the genotypes in the field.

Table 3. PCR fragment sizes performed for 6 SSR markers and their PIC and PD values

SSR Marker	Observed product sizes	Number of alleles	Allele sizes	PIC	DP	MI	HE*
NGSaf-69	304-332	6	304, 309, 310, 318, 324, 332	0.6694	0.2864	0.6778	0.6778
NGSaf-94	398-408	4	398, 400, 402, 408	0.6691	0.6774	0.8981	0.8981
NGSaf-130	380-390	5	380, 383, 386, 388, 390	0.6529	0.5897	0.8183	0.8183
NGSaf-145	376-380	3	376, 378, 380	0.6395	0.2623	0.5886	0.5886
NGSaf-238	380-396	6	380, 384, 386, 390, 394, 396	0.6778	0	0	0
SES-85	208-214	4	208, 210, 212, 214	0.6778	0	0	0

*PIC: Polymorphic information content, DP: Discriminating power, MI: Marker index, HE: Heterozygosity index

a)



b)

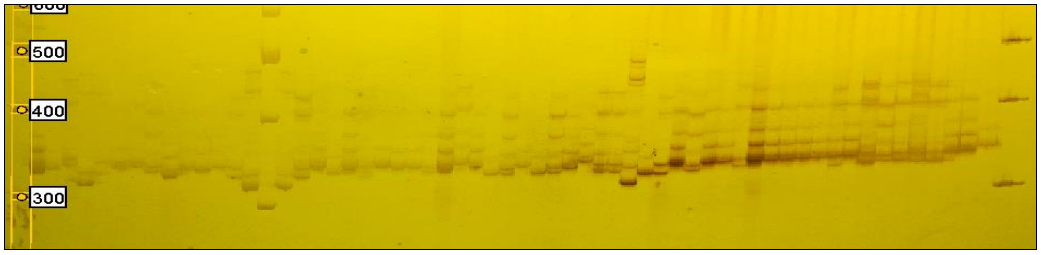


Figure 1. PCR products of SSR marker NGSaf-238 (a), NGSaf-69 (b) amplified from DNA of 59 safflower genotypes and resolved in a 6% polyacrylamide gel.
Lanes M: 50 bp DNA ladder, respectively. Lane 1: 1 (♂), Lane 2: 3 (♀), Lane 3: 10 (♂), Lane 4: 21 (♀), Lane 5: 25 (♀), Lane 6: 28 (♀), Lane 7: 29 (♂), Lane 8: 30 (♂), Lane 9: 35(♂), Lane 10: 35 (♂), Lane 11: Dinçer (♀), Lane 12: Balcı (♂), Lane 13: Linas (♂), Lane 14: Olas (♂), Lane 15-38: Hybrid BA1- Hybrid BA24, Lane 39-59: Hybrid EC1- EC21

Table 4. Genetic parameters of 6 SSR primers used in the study

SSR Marker	Size	Fit	Fst	ne	I	H_o	H_e	H_a*
NGSaf-69	118	-0.174	0.413	4.533	1.618	0.915	0.786	0.458
NGSaf-94	118	0.294	0.647	2.845	1.191	0.458	0.654	0.229
NGSaf-130	118	0.560	0.780	2.902	1.160	0.288	0.661	0.144
NGSaf-145	118	0.297	0.648	2.516	0.993	0.424	0.608	0.212
NGSaf-238	118	-0.072	0.464	3.119	1.289	0.729	0.685	0.364
SES-85	118	0.301	0.650	3.115	1.254	0.475	0.685	0.237
Mean	118	0.187	0.593	3.172	1.251	0.548	0.680	0.274
St. Dev.	-	-	-	0.702	0.207	0.230	0.059	0.115

*ne = Effective number of alleles (KIMURA and CROW, 1964; EWENS, 2016), I: Shannon's information index (KONOPIŃSKI, 2020), H_o: Observed Heterozygosity, H_e: Expected Heterozygosity (unbiased) (NEI, 1987), H_a: Average Heterozygosity

Table 5. Fixation index (Fis) (Wright et al., 1978) as a measure of heterozygote deficiency or excess

Allele \ Locus	NGSaf-69	NGSaf-94	NGSaf-130	NGSaf-145	NGSaf-238	SES-85
Allele A	-0.103	0.186	0.722	0.288	-0.167	0.107
Allele B	-0.054	0.441	0.409	0.113	-0.087	0.277
Allele C	-0.181	0.639	0.514	0.656	-0.103	0.353
Allele D	-0.304	0.184	-0.009	****	0.190	0.374
Allele E	-0.124	****	1	****	-0.008	****
Allele F	-0.083	****	****	****	-0.008	****
Total	-0.174	0.294	0.560	0.297	-0.073	0.301

**** Not calculated.

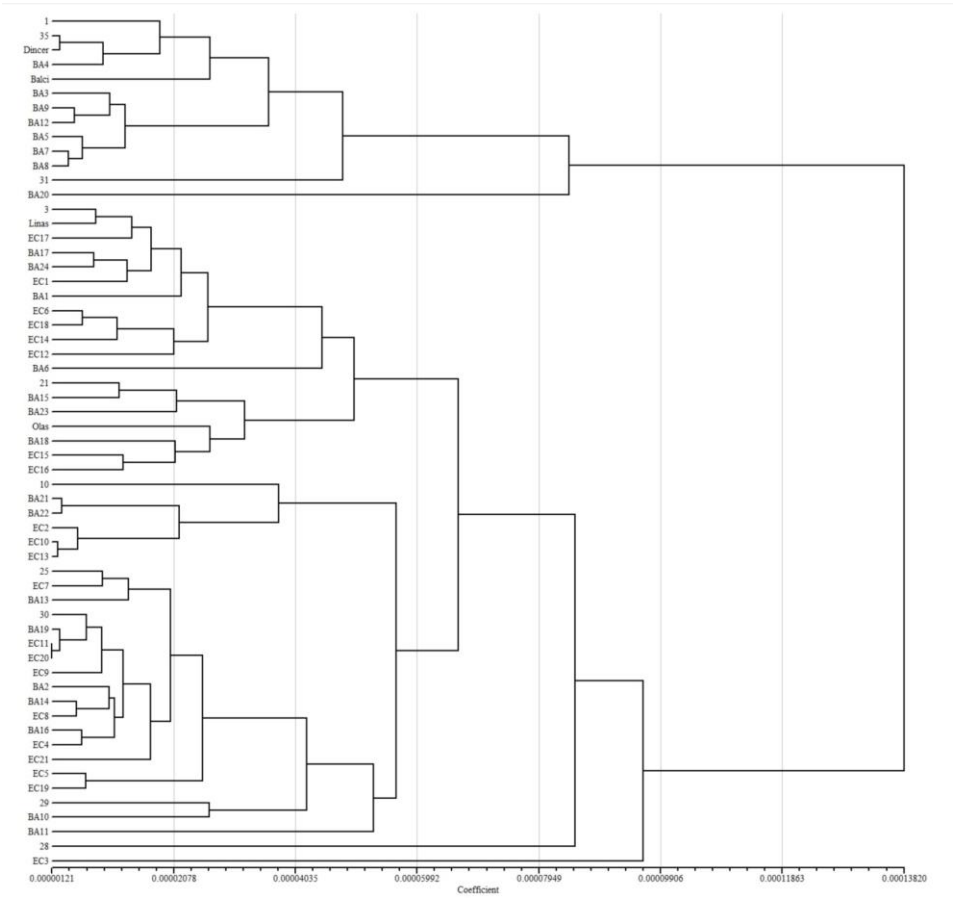


Figure 2. Dendrogram of 59 safflower genotypes based on UPGMA cluster analysis and Jaccard's similarity coefficients using allelic diversity data for 6 SSR markers.

The dendrogram in Figure 2 was created in order to understand better the genetic relationships of the parents and hybrids used in the study, as compared to the molecular markers. The dendrogram analysis revealed at least four possible major clusters in the parents and hybrids. Several sub-clusters within the major clusters were not clearly observable. Safflower cultivars of registered in Turkey were distributed across all four clusters and the accessions from USDA were defined in most of the clusters. The dendrogram based method analysis revealed two major clusters which corresponded to spiny and spineless safflower genotypes. These data showed no relation between genotypic clusters and geographical origin and generally clustered spiny safflower genotypes into the same clade.

CONCLUSION

In this study, inter-varietal SSR allelic diversity was performed in a panel of 59 safflower genotypes, which included safflower cultivars registered of in Turkey cultivars and the accessions from USDA using 10 SSR markers. In this study, a high average number of alleles (4.67) and high average PIC values (0.664) across 59 accessions were observed, which suggesting that safflower may possess a high level of SSR polymorphism. Studies have shown that there is confirmed a wide variation between average allele numbers and polymorphic information content values. The reason for this variation can be due to the diversity of safflower genotypes from different geographic regions and the number of SSR markers. It is suggested that the studied 6 SSR markers could be utilized in safflower breeding studies based on molecular analysis.

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GENETIČKI DIVERZITET GENOTIPOVA ŠAFRANIKA (*Carthamus tinctorius* L.) DOBIJEN HIBRIDIZACIJOM KORIŠĆENJEM SSR MARKERA

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Izvod

Šafranik je jednogodišnja uljarica koja u svetu ima zdravo jestivo ulje koje sadrži veliku količinu nezasićenih masnih kiselina. U ovoj studiji smo istraživali registrovane sorte šafranike Turske (4), genotipove preuzete iz USDA (10) i njihove hibride (45) za genetsku varijaciju koristeći 10 lokusa ponavljanja jednostavne sekvence (SSR). Genetski diverzitet izračunat je registrovanim sortama, genotipovima i hibridima na sledeći način: srednji broj alela (4,67), očekivana heterozigotnost (0,680), prosečni efektivni broj alela (3,172) i sadržaj informacija o polimorfizmu (0,664). Analiza dendrograma je otkrila najmanje četiri moguća glavna klastera kod roditelja i hibrida. Visok nivo genetske raznovrsnosti objašnjen između populacija i Fst izračunat (0,593) sugeriše da su klasteri međusobno diferencirani. Registrovane sorte šafranike iz Turske bile su raspoređene u sva četiri klastera, a u većini klastera definisani su genotipovi iz SAD. Analiza metoda zasnovana na dendrogramu otkrila je dva glavna klastera koji su odgovarali genotipovima šafranike sa bodljima i bez. Predloženo je da se proučavanih 6 SSR markera može koristiti za studije oplemenjivanja šafranike na osnovu molekularne analize.

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