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Assessment of genetic diversity in safflower cultivars and breeding lines with AFLP markers

Aspir çeşit ve ıslah hatlarında AFLP belirteçleri ile genetik çeşitliliğin belirlenmesi

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ABSTRACT

Safflower is a self-pollinating oilseed crop and has been cultivated since antiquity in the Middle East. Safflower has become a minor oilseed crop, and to increase its cultivation it is necessary to characterize and introduce new germplasm. Thirty-eight safflower cultivars and breeding lines from different countries have been screened with 8 AFLP primer combinations to assess genetic diversity and relationships among these genotypes. AFLP primers produced total of 410 bands, 113 of which was polymorphic, with an average of 51.2 bands per assay. Polymorphism ratio changed between 14.8 to 41.4% among the genotypes. Polymorphism information content (PIC) and resolving power (Rp) of the primer combinations were between 0.21-0.38 and 5.11-27.68, respectively. Genetic similarity coefficients were between 0.245-0.850 with an average of 0.573. Dendrogram based on similarity matrix produced three clusters and three cultivars clustered separately from the rest of the genotypes. The study shows genetic variation within germplasm and could be useful for breeding and genetic diversity studies in the future.

Key Words: *Carthamus tinctorius*, Genetic diversity, Molecular markers, Safflower

ÖZ

Aspir, kendine döllenen tek yıllık bir yağ bitkisidir ve antik çağlardan beri Orta Doğu'da yetiştirilmektedir. Aspir önemini yitiren bir kültür bitkisi haline gelmiştir ve aspir tarımını artırmak için yeni çeşitlerin temini ve karakterizasyonu gereklidir. Farklı coğrafi kökene sahip 38 aspir çeşidi ve ıslah hattı arasındaki genetik çeşitliliği ve ilişkilerini belirlemek için 8 AFLP primer kombinasyonu kullanılarak genotiperler taranmıştır. AFLP primerleri, reaksiyon başına ortalama 51.2 bant ve toplamda 410 bant üretmiştir. Üretilen bantların 113 tanesinin genotipler arasında polimorfik olduğu gözlenmiş ve polimorfizm oranı %14.8-%41.4 arasında değişmiştir. Primer kombinasyonlarının polimorfizm bilgi içeriği (PIC) 0.21-0.38 ve çözümleme gücü (Rp) 5.11-27.68 arasında değişmiştir. Aspir çeşit ve ıslah hatları arasındaki genetik benzerlik katsayıları 0.245-0.850 arasında değişkenlik gösterirken; ortalama genetik benzerlik 0.573 olarak bululnumuştur. Benzerlik matrisine dayalı dendogram, genotipleri üç küme içine yerleştirmiş fakat üç kültivar gruplandırılamamıştır. Mevcut çalışma, genotipler içerisindeki genetik çeşitliliğin boyutunu göstermekte ve gelecekte yapılacak ıslah ve genetik analizler için faydalı olacaktır.

Anahtar Kelimeler: *Carthamus tinctorius*, Genetik çeşitlilik, Moleküler Belirteç, Aspir

Introduction

The genus *Carthamus* belongs to the *Asteraceae* family and contains 25 species, most of which are native to the Mediterranean basin. Safflower (*Carthamus tinctorius* L.) is the only cultivated species of the genus with a diploid chromosome number of 2n=24, and has been cultivated for 4000 years (Mündel and Bergman, 2009). Safflower is mainly grown as an oilseed crop and seeds contain 25-40% crude oil, but it has other uses, such as dye production from flowers for food industry and as a fodder crop for livestock (Sirinivas et al., 1999; Landau et al., 2004). Safflower oil is also used clinically for the treatment of various illnesses and contains high oleic and linoleic acids contents, essential for human diets (Weiss, 2000; Kim et al., 2007).

Safflower is a self-pollinating plant, and breeding activities were based on genetic variability existed within cultivars and local sources collected from different parts of the world (Mündel and Bergman, 2009). Genetic diversity could be assessed using information on morphology, agronomic variables and molecular biology of the species. Although each method has its own advantages, molecular tools provide more polymorphisms to reveal existing diversity in a species (Tanksley and McCouch, 1997). Different molecular marker systems exist and they have been used to investigate genetic diversity in plant species, but PCR-based methods are preferred, because they produce more polymorphisms and easier to implement and use (Ragot and Hoisington, 1993).

Molecular tools have been used to analyze species delamination, domestication, breeding and seed purity (Chapman and Burke, 2007; Naresh et al., 2009; Pearl et al., 2014) in safflower. Genetic diversity of safflower genotypes have also been studied with different marker systems, such as RAPD (Amini et al., 2008; Giachino and İnan, 2019), ISSR (Ali et al., 2020), SRAP (Peng et al., 2008), AFLP (Sehgal and Raina, 2005; Johnson et al., 2007; Sehgal et al., 2009), SSR (Mokhtari et al., 2018) and peroxidase gene-based polymorphisms (POGP) markers (Çankaya et al., 2019; Yıldız et al., 2022). Genetic diversity studies with molecular markers may provide valuable insights into relationships between different safflower genotypes for breeding studies.

The safflower genotypes used in the present study have been characterized for their morphological and agronomic parameters (Tonguç and Erbaş, 2009) and with the POGP markers (Çankaya et al., 2019). The aim of the present study was to evaluate genetic diversity among the same safflower genotypes with AFLP markers to asses relationships between these genotypes.

Material and Methods

Plant material

Seven safflower breeding lines and 31 cultivars originating from 6 countries were used in the study. Plants with PI numbers were obtained from USDA Western Regional Plant Introduction Station (Pullman, WA, USA) and local safflower cultivars were obtained from Ege Agricultural Research Institute (Menemen, İzmir, Türkiye) (Table 1).

DNA isolation

Seeds were sown in plastic pots for germination, and leaves from seedlings were collected and stored at -80 °C until used. DNA isolation was carried out using CTAB method according to Doyle and Doyle (1990) with minor modifications. Pellets were dissolved in TE buffer and the final concentration of DNA was adjusted to 250 ng μL^{-1} with a spectrophotometer and stored at -20 °C for later use.

AFLP analysis

AFLP analysis system I kit was used for digestion, pre-selective and selective amplifications following the manufacturer's instructions (Invitrogen Life Technologies, USA). Genomic DNA (250 ng) of safflowers was digested with *Eco*RI and *Mse*I restriction enzymes at 37 °C

Table 1. Safflower cultivars and breeding lines used to assess genetic diversity within cultivated safflower germplasm

Gene bank no	Genotype name	Origin	Breeding status	Gene bank no	Genotype name	Origin	Breeding status
PI 537110	Quiriego 88	Mexico	Cultivar	PI 560177	Oleic Leed	USA	Cultivar
PI 537111	Sahuaripa 88	Mexico	Cultivar	PI 538779	Centennial	USA	Cultivar
PI 561703	San Jose 89	Mexico	Cultivar	PI 601506	S-517	USA	Cultivar
PI 572475	Saffire	Canada	Cultivar	PI 572472	Rehbein	USA	Cultivar
PI 592391	AC Sunset	Canada	Cultivar	PI 525458	Finch	USA	Cultivar
PI 559909	AC Stirling	Canada	Cultivar	PI 572436	Leed	USA	Cultivar
PI 603206	Lesaf 414	Canada	Breeding line	PI 508098	Hartman	USA	Cultivar
PI 610263	Enana	Spain	Breeding line	PI 537695	Ole	USA	Cultivar
PI 657792	Rinconada	Spain	Cultivar	PI 572434	UC-1	USA	Cultivar
PI 657797	CH-353	Spain	Breeding line	PI 572414	US-10	USA	Cultivar
TR 69497	Dinçer 5-18-1	Türkiye	Cultivar	PI 572471	Sidwill	USA	Cultivar
TR 69498	Yenice 5-38	Türkiye	Cultivar	PI 537694	Royal	USA	Cultivar
TR 69499	Remzibey-05	Türkiye	Cultivar	PI 537692	Gila	USA	Cultivar
PI 538025	Montola 2000	USA	Cultivar	PI 525457	Girard	USA	Cultivar
PI 601166	Oker	USA	Cultivar	PI 514632	Ziyang	China	Cultivar
PI 572465	4022	USA	Breeding line	PI 514631	Yuyao	China	Cultivar
PI 572439	PCA	USA	Breeding line	PI 514624	Shufu	China	Cultivar
PI 572421	Frio	USA	Cultivar	PI 514620	Huaxian	China	Cultivar
PI 572418	Arizona Saf Com III	USA	Breeding line	PI 506426	FO-2	China	Cultivar

for 2 h and the reaction was terminated by raising temperature to 70 °C for 10 min. DNA samples were ligated to *Eco*RI and *Mse*I adaptors for 2 h at 20 °C with T4 DNA ligase and the samples were diluted with TE buffer for pre-selective amplifications. Pre-selective amplifications were performed with *Eco*RI+A and *Mse*I+C primers with 20 cycles of 94 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s. Pre-amplified fragments were diluted 1:50 with TE buffer for use in selective PCR amplifications. For selective amplifications, 5 µl of diluted PCR product, 0.5 µl of *Eco*RI and 4.5 µl of *Mse*I primers (primers with 3 selective nucleotides), dNTP mix, 2 µl of 10X PCR solution, 7.9 µl of sterile distilled water and 0.1 µl of *Taq* polymerase were used per reaction. The touchdown method was used for PCR amplifications. Selective PCR amplifications were carried out as described by Tonguç et al. (2011). Primers D700 and D800 and their nucleotide sequences were given by Johnson et al. (2007).

PCR products were separated in 6% denaturing polyacriylamide (19:1 acrylamide: bisacrylamide) sequencing gel. Electrophoresis was carried out using Owl S4S gel electrophoresis system (Thermo Scientific, USA) for 3 h in 0.5X TBE buffer at 2400 V with temperature of 50 °C. Gel preparation, denaturation and silver staining procedures were carried out as described by Pillen et al. (2000).

Data analysis

Dried gels were scanned and scored for presence (1) and absence (0) of bands. For each primer combination, total number of bands, number of polymorphic bands, polymorphism ratio (%), polymorphism information content (PIC) and resolving power (Rp) was calculated. PIC value was calculated with the following formula, PIC = 2fi (1-fi) where fi represents the presence of loci and (1-fi) represent the absence of loci of a molecular marker (Roldan-Ruiz et al., 2000). Rp value for each primer combination was calculated according to Prevost and Wilkinson (1999). Rp = Σ Ib where Ib represents band informativeness and calculated as Ib = 1 - [2x (|0.5-p|)] and p represents proportion of genotypes containing the band. Data matrix was analyzed with NTSYS-pc (ver. 2.2) and pair-wise similarities were calculated using Jaccard's similarity coefficients with SIMQUAL subprogram (Rohlf, 1992). Dendogram was constructed using the SAHN subroutine and the unweighted paired group method using arithmetic averages (UPGMA). Mantel's z test values were calculated using the MX COMP in the NTSYS-pc to determine the degree of compatibility between dendogram created by the UPGMA method with the similarity matrix.

Results and Discussion

In the present study, total of 38 cultivars and breeding lines were used to investigate genetic diversity among safflower genotypes. Three cultivars/lines from Turkey and Mexico, 4 from Canada, 3 from Spain, 5 from China and 19 from USA represented cultivated safflower germplasm (Table 1). Eight AFLP primer combinations were used to amplify genomic DNA and these primers amplified 410 bands among the cultivars and lines. Number of polymorphic bands produced by the AFLP primers was between 4-31. The highest number of polymorphic bands were produced by D800 (31) and D700 (29) primers, while the fewest number of polymorphic bands were produced by EACA/MCTT (4) primer (Table 2). Polymorphism ratio of the primer combinations were between 14.8-41.4% among the safflower cultivars and lines. The highest polymorphisms ratios were produced by D700 and D800 primers, while the lowest polymorphism ratio was detected in EACA/MCTT. AFLP primers, along with other molecular marker systems, have been used to asses genetic diversity within the safflower germplasm (Sehgal and Raina, 2005; Johnson et al., 2007; Sehgal et al., 2009). These studies showed that AFLP markers produced more total and polymorphic bands and higher polymorphisms ratios compared to RAPD and ISSR markers used in these studies. Among the 14 safflower cultivars, 4 AFLP primers yielded between 16-23 polymorphic bands and polymorphism ratio of 41.0-72.4%. The average number of polymorphic bands produced by each marker type was found to be 2.4 for RAPD, 1.3 for ISSR and 20.5 for AFLP (Sehgal and Raina, 2005). When 85 safflower accessions were assayed with 10 AFLP primers, total number of bands were between 20-52, number of polymorphic bands were between 10-39 with polymorphism ratio of 50.0-88.8 with an average of 70.4% (Sehgal et al., 2009). These results showed that average polymorphism ratio for AFLP markers was higher among the accessions, and breeding activities reduced number of polymorphic loci to 27.5% observed in the present study and 20.5% reported by Sehgal and Raina (2005). Reduction of polymorphism among the breeding material was also noticed by Johnson et al. (2007). The results also showed that primer combinations and number of cultivars affect number of bands produced and polymorphism ratios.

PIC values of the markers depend on the number of alleles and also allele distribution among the assayed genotypes and change between 0.0-0.5 for dominant markers (Roldan-Ruiz et al., 2000). PIC value of AFLP markers in the study was between 0.21-0.38 with an average PIC value of 0.29. Even though primer EACA/MCTT produced the fewest number of bands and polymorphic bands, it had the highest PIC value, showing distribution of polymorphic bands greatly affects observed PIC values. Çankaya et al. (2019) reported average PIC value of 0.26 for the same genotypes using POGP markers, which was close to reported value for AFLP markers for the same genotypes used in this study. Average PIC value of 10 AFLP markers was found to be 0.30 for safflower accessions (Sehgal et al., 2009).

Table 2. AFLP primers used in the study and the results of amplifications and evaluated parameters

Primer combination	Total number of bands	Number of polymorphic bands	Polymorphism ratio (%)	PIC	Rp
EACA/MCTT	27	4	14.8	0.38	5.11
EACT/MCTT	53	8	15.1	0.30	12.95
EAGG/MCAC	35	7	20.0	0.31	6.79
EAGC/MCAT	51	13	25.5	0.21	15.84
EAGC/MCAA	61	11	18.0	0.37	12.53
EAGC/MCAG	41	10	24.4	0.27	7.84
D700	70	29	41.4	0.25	25.05
D800	76	31	40.8	0.30	27.68
Total	410	113	-	-	-
Mean	51.2	14.1	27.5	0.29	14.22

Different marker systems have also been used to explore genetic diversity within safflower germplasm. PIC values for dominant markers, such as RAPD and ISSR markers were found to be 0.38 and 0.14 (Seghal et al., 2009; Giachino and İnan, 2019), 0.19 and 0.44 (Seghal et al., 2009; Ali et al., 2020), respectively. These results show that PIC value change depending on marker systems and germplasm used. Rp values of the AFLP primers was between 5.11-27.68 with an average of 14.22 and the primers EACA/MCTT had the lowest and D800 had the highest Rp values, respectively. Rp values of AFLP markers were 40.62 and 59.16 among the safflower cultivars and accessions, respectively (Seghal and Raina, 2005; Seghal et al., 2009) and our values for the AFLP markers were lower than the reported values for AFLP markers.

The similarity matrix for all cultivars and lines was calculated using the Jaccard's coeficient with NTSYS-pc program. The calculated similarity coefficients found to be between 0.245-0.850. The lowest values obtained were 0.245 between UC-1 and Dinçer 5-18-1 and 0.26 between UC-1 and Girard. The highest similarity coefficient was 0.850 between Gila and US-10. The average of the coefficient values of all varieties and lines was found to be 0.573. In order to reveal the relationships between the genotypes, a dendogram was constructed with NTSYS-pc program using UPGMA method (Figure 1). Dendogram showed that UC-1 was not included in any cluster, and Girard and Arizona Safflower Composite III clustered independently from the main group. Oleic Leed and CH-353 formed a cluster together independent from the rest of the genotypes. The rest of the safflower genotypes formed two separate clusters. The smaller cluster contained Montola 2000, FO-2, Remzibey-05, Enana, Dinçer 5-18-1 and Yenice 5-38. Genetic similarity between Remzibey-05 and Dinçer 5-18-1 was 0.62, between Remzibey-05 and Yenice 5-38 was 0.55 and between Yenice 5-38 and Dinçer 5-18-1 was 0.64. Yenice 5-38 was the first composite safflower variety released in Türkiye and therefore it might be used as progenitor for the selection of other local safflower varieties (Köse, 2017). Due to their high genetic similarity and possible similar genetic background they were clustered very close to each other. The other cluster contained 27 cultivars and breeding lines. AFLP primer combinations employed in the study did not produce enough polymorphism to distinguish Gila and US-10 from each other, and these two cultivars were grouped together. Mantel's z test values were calculated to determine the degree of compatibility between the dendogram produced by the UPGMA method with the corresponding similarity matrix. The obtained cophenetic correlation coefficient value ($r = 0.83$) was found to be significant, indicating there is a good fit between the dendogram and the similarity matrix.

The dendogram produced by AFLP markers in this study was different from the dendogram produced by POGP markers for the same safflower genotypes (Çankaya et al., 2019). Cultivars originating from Türkiye clustered close to each other and were in the same group with AFLP markers, however they were clustered very differently when POGP makers used, indicating these marker systems use different sequences to amplify. Therefore, they may cause incongruence between the dendograms. Sehgal and Raina (2005) and Sehgal et al. (2009) obtained different dendograms for the same genotypes using RAPD, ISSR and AFLP markers for safflower. Such phenomenon was also reported for other crop species, such as maize (Pejic et al., 1998) and cashew (Archak et al., 2003) where different marker types have been employed with the same genotypes. It was suggested that self-pollination, selection and inbreeding could cause incongruity as well as the type of markers used to amplify genomic regions (Powell et al., 1996; Sehgal and Raina, 2005; Johnson et al., 2007). Mean genetic similarity was found to be high in the present study. Safflower is predominantly a self-pollinating species and the plant material used in the study consisted of cultivars and breeding lines, which are highly inbred. It was observed that genetic variation was lower in cultivars and

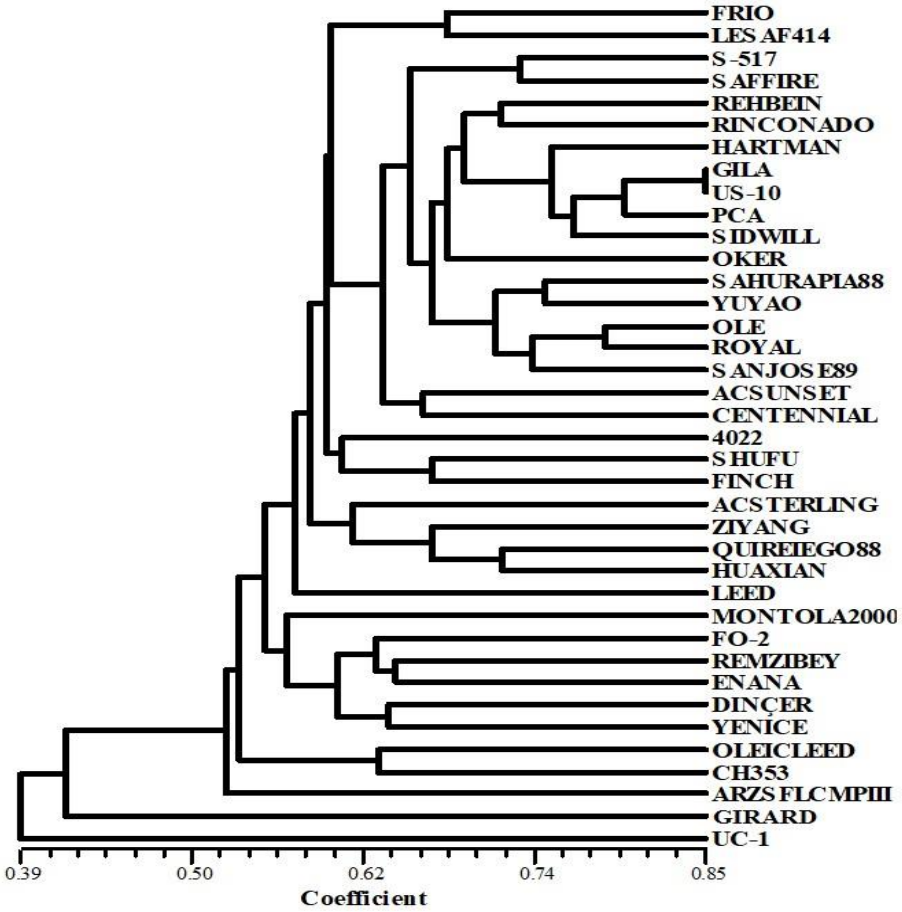

Figure 1. UPGMA dendogram of 38 safflower cultivars and breeding lines produced by 8 AFLP primer combinations

breeding lines than that of accessions for safflower (Sehgal and Raina, 2005; Johnson et al., 2007). Another study revealed that a subset of a RIL population derived from a cross between Dinçer 5-18-1 and Remzibey-05 showed a polymorphism ratio of 4.5% detected by AFLP markers (Tonguç et al., 2011), indicating that these safflower cultivars are highly similar as revealed by cluster analysis.

Conclusion

The present study evaluated genetic relationships among 38 safflower cultivars and breeding lines with AFLP markers. AFLP primer combinations differed vastly for their ability for amplification of polymorphic bands. Number of bands produced by AFLP markers, polymorphism ratio and PIC value was similar to reported average values for AFLP markers. Dendogram based on similarity matrix and genetic similarity coefficients showed that safflower cultivars and lines were genetically close to each other suggesting the need to introduce genetic variation into safflower germplasm to create variability for breeding studies.

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Author contributions: M.T. investigation, writing-review and editing; S.E. investigation

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