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Identification of 26 Germplasms of Safflower (*Carthamus tinctorius* L.) with ISSR and SCAR Markers

Jung Sook Sung*, Gyu Taek Cho*, Suk Young Lee*, Hyung-Jin Baek*, So Hye Park**, and Man Kyu Huh**†

*National Academy of Agricultural Science, RDA, Suwon 441-100, Korea
**Department of Herbal Crop Research, NIHHS, RDA, Eumseong 369-873, Korea

ABSTRACT Safflower (*Carthamus tinctorius* L.) is a herb primarily distributed throughout in the world. We have used the inter-simple sequence repeats (ISSR) technique to investigate the phylogenetic relationships and genetic diversity of *C. tinctorius*. Of all germplasms, 88.7% were polymorphic among all germplasms. Mean genetic diversity within germplasms was very low (0.048). The Turkey germplasm had the highest expected diversity (0.082) and Australia germplasm was the lowest (0.020). These values indicate that most of the genetic diversity of safflower is found among germplasms and there is a high among-germplasm differentiation. We found eight phenetic bands for determining the specific marker of germplasm with SCAR markers. The regions of the Mediterranean Sea and India may be the most probable candidates for the origin of safflower. The tree showed four major clades: (1) European germplasms, (2) Azerbaijan, Egypt, and Ethiopia, (3) Australia, and (4) America.

Keywords : safflower (*Carthamus tinctorius* L.), ISSR, SCAR, phylogenetic relationships

Safflower (*Carthamus tinctorius* L.) is one of humanity's oldest crops (Johnston *et al.*, 2002). Chemical analysis of ancient Egyptian textiles dated to the Twelfth dynasty identified dyes made from safflower, and garlands made from safflower were found in the tomb of the pharaoh Tutankhamen. Safflower is most commonly known as 'kusan' (India, Pakistan), 'suff' (Ethiopia), 'Le carthame' (France), and 'honghua' (red flower) in China and Korea.

Traditionally, safflower was used for favoring foods, medicines, making red (carthamin), and yellow dyes, especially before cheaper aniline dyes became available (Zohary & Hopf, 2000).

Inter-simple sequence repeats (ISSR) amplification is a technique which can rapidly differentiate between closely related individuals (Zietkiewicz *et al.*, 1994). The ISSR technique involves anchoring of designed primers to a subset of simple sequence repeats (SSRs) and amplifies the region between two closely spaced, oppositely oriented SSRs (Hamada & Kakunaga, 1982).

Simple sequence repeats occurs as tandem repeats of di-, tri-, and penta-nucleotides and are ubiquitous in eukaryotic genomes (Gupta *et al.*, 1994). These repeats have been found interspersed in various combinations of two or more ISSRs (compound ISSRs) as well as with the other single and multicopy sequences (Hamada *et al.*, 1984; Martinez-Soriano *et al.*, 1991). They were often singled out from other sequences of interest because of their association with a high level of polymorphism (Luty *et al.*, 1990; Morral *et al.*, 1991). Of the most studied repeats, (GT)n, (GA)n, (GATA)n and (GACA)n, the copy number of (GT)n per haploid genome varies from 100 copies in yeast to 100,000 copies in mouse genome (Stallings *et al.*, 1991). A high level of ISSR-associated polymorphism has been observed in plants (Poulsen *et al.*, 1993; Schmidt *et al.*, 1993).

The sequence characterized amplified regions (SCAR) varies with the repeat sequence-order in primers. Markers that are polymorphic between the pools will be genetically linked to the loci determining the trait used to construct the pools (Khampila *et al.*, 2008). The transformation of these markers such as SCAR is generally the next step before their routine application in marker-association selection (Paran & Michelmore, 1993).

Despite considerable work in germplasm collection and evaluation, the group was strongly of the opinion that

†Corresponding author: (Phone) +82-51-890-1529
(E-mail) mkhuh@deu.ac.kr <Received September 20, 2010>

germplasm needs had a very high priority Knowles, 1981). Germplasm characterization is an important link between the conservation and utilization of plant genetic resources. Molecular DNA techniques allow researchers to identify accessions at the taxonomic level, to assess the relative diversity within and among species, and to locate diverse accessions for breeding purposes. Moreover, the commercial value associated with identifying useful traits places a direct value on GenBank ensuring the long-term preservation of a collection.

Studies to clarify the overall status of several countries of this species were not conducted. Thus, the aim of this paper was to determine the compositions of the components of *C. tinctorius* for the first time and to compare it with the composition of *C. tinctorius* from the other countries. This study was carried out to examine 26 germplasms of *C. tinctorius* in order to evaluate genetic diversity and population structure in this species.

MATERIALS AND METHODS

Plant materials

Wild seeds were collected from 149 germplasms of *C. tinctorius* of 26 countries including Korea (Table 1). All seeds of samples were obtained from National Agrobiodiversity Center, NAAS, PDA (Suwon, Gyeonggi Province in Korea).

Randomly selected seeds per 149 germplasms were sown into outdoor plots with same environmental conditions. We found that 149 populations of *C. tinctorius* were same

Table 1. Code of the genus *Carthamus tinctorius* L. (Compositae) in this study.

Code	Country	Region or Site
AFG	Afghanistan	Kabul
ARM	Armenia	Alex Manoogian, Erevan
AUS	Australia	Melbouren, Victoria
AZE	Azerbaijan	Ganja, Ganja-Qazkh
CAN	Canada	Winnipeg, Manitoba
CHN	China	Ching Tao, Sandong
EGY	Egypt	Heliopolis, Cairo
ETH	Ethiopia	Addis Ababa
HUN	Hungary	Biatorbagy, Budapest
IND	India	Pondicherry, Tamil
IRN	Iran	Meshed
KAZ	Kazakhstan	Semipalatinsk, Vostochno-Kazakhstanskaya Oblast
KOR	Korea	Suwon, Gyonggi-do
MAR	Morocco	Rabat, Rabat-Sale-Zemmour-Zaer
MEX	Mexico	Leon, Guanajuato
PAK	Pakistan	Kakapir, Karachi
RUS	Russia	Omsk Oblast
SDN	Sudan	Khartoum, Khartoum State
SYR	Syria	Alepo, Jabal Semaan
TKM	Turkmenistan	Anau, Ahal Province
THA	Thailand	Ratchasima, Nakhon
TJK	Tajikistan	Khujand, Sughd
TUR	Turkey	Antakya, Hatay
UKR	Ukraine	Mariupol, Donetsk
USA	United States of America	State College, PA
UZB	Uzbekistan	Termiz, Surxondaryo Viloyati

lineages or accessions. Thus we selected the representative one germplasm per nation in the world and analyzed 50 seedling plants from each origin.

DNA extraction

We randomly selected ten plants per germplasm for the analyses within and among germplasms. Genomic DNA was isolated from one unexpanded leaf of one plant. DNA was extracted with the plant DNA Zol Kit (Life Technologies Inc., USA) according to the manufacturer's protocol. Extracted DNA was quantified using a DyNA 200 fluorimeter (Amersham Pharmacia Biotech, Italy) according to the manufacturer's instructions.

ISSR analysis

ISSR-PCR analysis was performed according to the protocol described by Charters *et al.* (1996) using one or two of the primers listed in Table 2. Fifteen arbitrarily chosen primers (Bioneer Technologies, Korea) were used. All the reactions were repeated twice and only reproducible bands were scored for analyses.

In brief, 10 ng of genomic DNA were added to a reaction mixture containing 1 unit of *Taq* polymerase, 15 mM $MgCl_2$ buffer, 0.2 mM of each dNTP and 0.2 M of primer. DNA was amplified on a thermocycler (Takara, Japan) using the following programme: 1 min at 94℃ and 30 cycles of (1 min at 94℃, 2 min at 55℃ and 30 sec at 72℃) followed by a final 10-min extension at 72℃.

A 100 bp ladder DNA marker (Pharmacia, USA) was used in the end of for the estimation of fragment size. The amplification products were separated by electrophoresis on 1.5% (w/v) agarose gels, stained with ethidium bromide, and photographed under UV light using Alpha Image TM (Alpha Innotech Co., USA).

SCAR analysis

To convert the selected ISSR band to a SCAR (sequence characterized amplified region) marker, the bands were excised, cloned and sequenced following the procedure outlined in Jung *et al.* (1999). The excised PCR bands were separated on 2.0% (w/v) agarose gels and purified using the QIAquick Gel Extraction Kit (QIAGEN, USA). The amplified fragments were cloned into a bluescript vector and sequenced using ABI Prism 377 Sequencer (Applied Biosystem, USA). The SCAR marker sequences were designed by identifying the original 10 bp sequence of the ISSR primer. The specific SCAR primer were used ten different individuals of same germplasms and 25 different bulk germplasms which were not shown the band. The 5.0 ul of amplification products were primarily

Table 2. List of decamer oligonucleotide utilized as primers, their sequences, and associated fragments.

No. of primer	Sequence(5'-3')	No. of fragments	Germplasm-specific bands	
			Band	Germplasm
ISSR-01	-$(GA)_{10}$-	12		
ISSR-02	-$(CA)_{10}$-	11		
ISSR-03	-$(AT)_{10}$-	12	ISSR-03-07	IND
ISSR-04	-$(TCC)_5$-	10		
ISSR-05	-$(CAC)_5$-	12	ISSR-05-04	KAZ
ISSR-06	-$(CAT)_5$-	10		
ISSR-07	-$(AGG)_6$-	10		
ISSR-08	-$(CGG)_6$ -	7	ISSR-08-03	IND
ISSR-09	-$(TGG)_6$-	9	ISSR-09-08, 09	ETH
ISSR-10	-$(CGT)_6$-	10		
ISSR-11	-$(GTG)_6$-	9	ISSR-11-02, 06	EGY
ISSR-12	-$(CAT)_6$-	8		
ISSR-13	-$(TGTC)_4$-	6	ISSR-13-01	TUR
ISSR-14	-$(ACTG)_4$-	7		

separated by electrophoresis on 2.0% (w/v) agarose gels, stained with ethidium bromide, and remainder 5.0 ul amplicon quantified the yield of total DNA using Bio-Rad (Bio-Rad Laboratories, Inc., USA) according to the manufacturer's instructions.

Statistical analyses

ISSR bands were scored by eye and only unambiguously scored bands were used in the analyses. Because ISSRs are dominant markers, they were assumed that each band corresponded to a single character with two alleles, presence (1) and absence (0) of the band, respectively. Loci were named based on the primer and observed band size.

The following genetic parameters were calculated using a POPGENE computer program (ver. 1.31) developed by Yeh *et al.* (1999): allele frequencies, the percentage of polymorphic loci (P_p), mean numbers of alleles per locus (A), effective number of alleles per locus (A_E), gene diversity (H), and Shannon's index of phenotypic diversity (Nei, 1973).

The estimation of genetic similarity (GS) between genotypes was based on the probability that an amplified fragment from one individual will also be present in another (Nei, 1973). GS was converted to genetic distance (1-GS). Homogeneity of variance among accessions was tested by Bartlett's statistics.

The Mantel test was examined the correlation between the matrix of genetic distance and spatial distance within a site. By use of allele frequencies, the probability that each genotype could arise independently was calculated following Parks & Werth (1993).

A maximum parsimony tree (MP) was inferred using heuristic search, branch-swapping options and tree bisection-reconnection. Confidence values for individual branches were determined by a bootstrap analysis with 1000 repeated sampling of the data. In addition, a phenetic relationship was constructed by the neighborjoining (NJ) method (Saitou & Nei, 1987) using the NEIGHBOR program in MEGA4 version 4.0 (Kumar *et al.*, 2008).

RESULTS

Band profiles generated by fourteen ISSR primers (Table 2) were screened for the 26 safflower germplasms in the world. There were 133 bands generated across all primers. Overall, there were 118 polymorphisms generated among the germplasms. Of all germplasms, 88.7% were polymorphic among all germplasms.

Across germplasms, the average number of alleles per locus (A) was 1.124, ranging from 1.052 to 1.203 (Table 3). The mean effective numbers of alleles per locus (A_E) was 1.083. Mean genetic diversity within germplasms was 0.048. In particular, the Turkey germplasm had the highest expected diversity (0.082); Australia germplasm, the lowest (0.020). Mean Shannon's information index (I) was 0.070, ranging from 0.030 to 0.120. The five highest genetic diversities among 26 countries were Turkey, Egypt, India, Kazakhstan, and Sudan (Table 3).

Total genetic diversity values (H_T) and interlocus variation in the within-germplasm genetic diversity (H_S) were 0.261 and 0.048, respectively (Table 4). Thus, the proportion of this diversity is not attributable to within germplasm diversity (H_S). On a per-locus basis, the proportion of total genetic variation due to differences among germplasms (G_{ST}) was 0.818. This indicated that about 18.2% of the total variation was within germplasms. These values indicate that most of the genetic diversity of safflower is found among germplasms and there is a high among-germplasm differentiation. The estimate of gene flow, based on G_{ST}, was very low among Korean germplasms of safflower / (N_m = 0.112).

Genetic identity based on the proportion of shared fragments was used to evaluate relatedness among species. The estimate of genetic identity ranged from 0.040 to 0.887 (data not shown). Thus, there were showed slight differences between germplasm-pairs.

The ISSR-05-04 band is the specific marker for the Kazakhstan germplasm, whereas no products were detected in individuals from other country germplasms (Fig. 1). The specific DNA fragment seemed to be useful to discriminate among germplasms and was used to develop the SCAR marker. Figure 2 gave rise to the yield of total DNA using Bio-Rad. We found many phenetic bands for determining the specific marker of germplasm with SCAR markers (Table 2). Eight bands (ISSR-03-07, ISSR-05-04, ISSR-08-03, ISSR-09-08, ISSR-09-09, ISSR-11-02, ISSR-11-06, and

Table 3. Measurements of genetic variation for 26 countries of *C. tinctorius*.

Country	Np	P_p	A	A_E	H	I	Rank
AFG	14	10.5	1.105	1.068	0.040	0.059	
ARM	11	8.3	1.083	1.067	0.036	0.052	
AUS	7	5.3	1.052	1.034	0.020	0.030	
AZE	21	15.8	1.158	1.103	0.060	0.089	
CAN	12	9.0	1.090	1.062	0.036	0.052	
CHN	13	9.8	1.098	1.058	0.035	0.053	
EGY	24	18.1	1.181	1.129	0.073	0.106	2
ETH	15	11.3	1.113	1.080	0.045	0.066	
HUN	14	10.5	1.105	1.069	0.040	0.059	
IND	23	17.3	1.173	1.125	0.070	0.102	3
IRN	18	13.5	1.135	1.074	0.046	0.071	
KAZ	21	15.8	1.158	1.123	0.067	0.097	4
KOR	11	8.3	1.083	1.063	0.035	0.050	
MAR	19	14.3	1.143	1.089	0.053	0.079	
MEX	7	5.3	1.053	1.030	0.019	0.028	
PAK	21	15.8	1.158	1.103	0.060	0.089	
RUS	10	7.5	1.075	1.060	0.032	0.047	
SDN	21	15.8	1.158	1.123	0.067	0.097	4
SYR	16	12.0	1.120	1.075	0.045	0.067	
TKM	18	13.5	1.135	1.094	0.053	0.078	
THA	8	6.0	1..060	1.034	0.021	0.032	
TJK	20	15.0	1.150	1.100	0.058	0.086	
TUR	27	20.3	1.203	1.146	0.082	0.120	1
UKR	16	12.0	1.120	1.103	0.055	0.077	
USA	10	7.5	1.075	1.052	0.030	0.044	
UZB	22	16.5	1.165	1.106	0.063	0.093	
Total	16.1	12.1	1.124	1.083	0.048	0.070	

Rank: The five highest genetic diversities among 26 germplasms.

Table 4. Estimates of genetic diversity of *C. tinctorius*. Total genetic diversity (H_T), genetic diversity within germplasms (H_S) proportion of total genetic diversity partitioned among germplasms (G_{ST}), and gene flow (N_m).

Total	H_T	H_S	G_{ST}	N_m
Mean	0.261	0.048	0.818	0.112

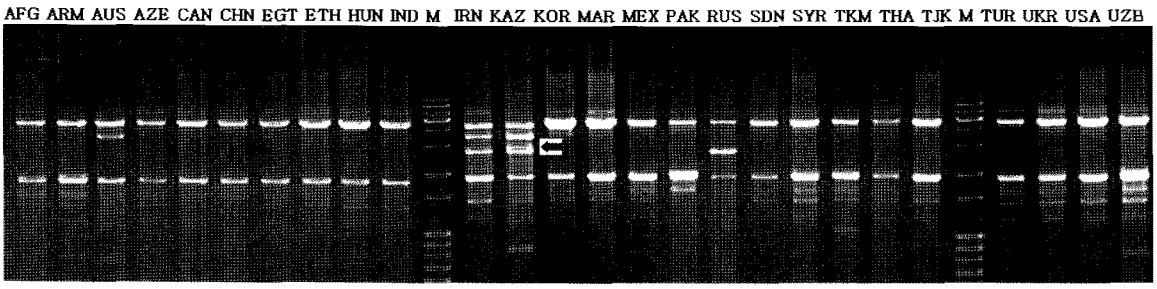

Fig. 1. The specific band of Kazakhstan (KAZ) with the ISSR-05 primer. Aberrations of AFG, ARM, AUS, and so on are the same as Table 1. M is the 100 bp ladder DNA marker.

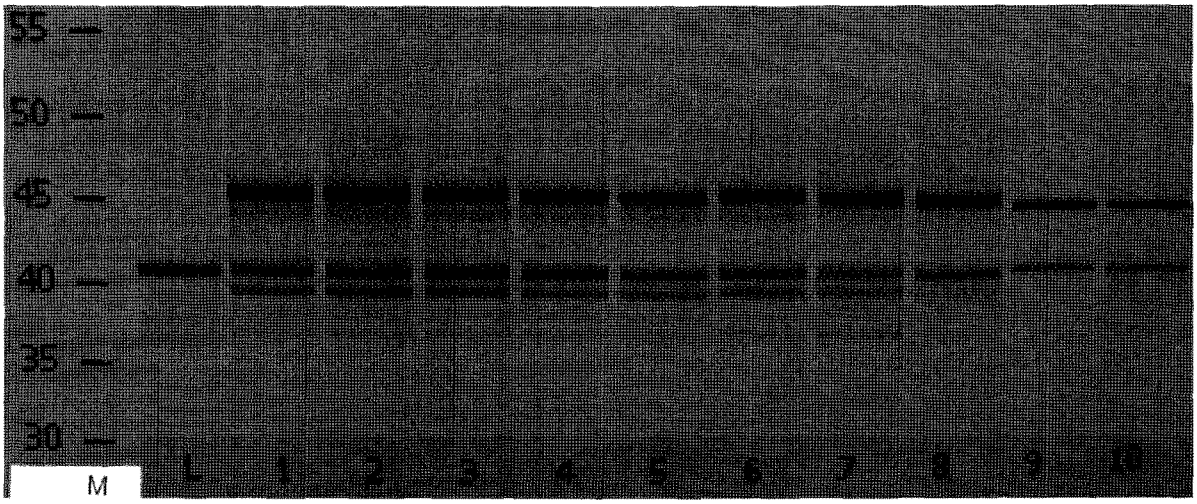

Fig. 2. The 13 different Kazakhstan individuals (1~10) were quantified specific bands for SCAR with ISSR-05 primer and other country bulk samples (L) is not amplified the SCAR primer. M: Marker DNA.

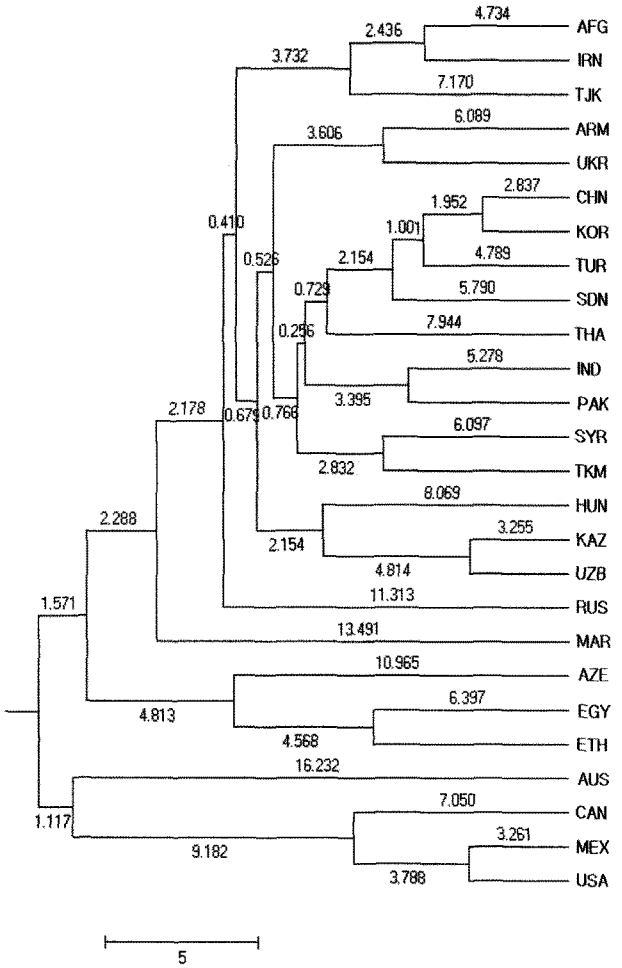

Fig. 3. A Phylogenetic tree for 26 germplasms of Carthamus tinctorius based on ISSR analysis.

ISSR-13-01) are specific for one germplasm.

Clustering of 26 safflower germplasms, using the NJ algorithm, was performed based on the matrix of calculated distances (Fig. 3). The phylogenic tree showed most germplasms were well separated each other. The tree showed four major clades: (1) European germplasms, (2) Azerbaijan, Egypt, and Ethiopia, (3) Australia, and (4) America. Asian countries were clustered within the larger European group. When a more worldwide selection of species was included, the tree also showed genetic differentiation among safflower species.

DISCUSSION

Genetic diversity among 26 selected germplasms of safflower was assessed with 133 ISSR bands. A low number of ISSR polymorphisms per primer were detected among germplasms. This finding concurs with previous studies in which low levels of polymorphism were detected among same samples on the basis of RAPD analysis (Huh personal communication, data not shown).

ISSR was a more efficient marker technique than RAPD for detecting polymorphism in these safflower germplasms in the world. A higher percentage (88.7%) of ISSR primers generated polymorphic bands within safflower germplasm compared with RAPDs (82.9%). This disparity reflected amplification patterns that were either monomorphic or too

faint to score. Another possible explanation for the differences in resolution of RAPDs and ISSRs is that the two marker techniques may target different portions of the genome (Karp *et al.*, 1996).

Vavilov (1950) proposed that crop species have centers of diversity that coincide with their centers of origin. Further, these centers should typically coincide with the distribution of the crop's nearest wild relatives. Harlan (1971) challenged the idea that centers of origin can be established for all crops and suggested that some crops may have "non-centric origins. Other authors (Doebley, 1990; Hancock, 2004) have also criticized Valvilov's concept and noted that trading patterns, farming practices, and environmental diversity can all influence the degree of diversity in a crop from a particular region. Although the origin and speciation time of safflower has not been known recently, region and time of origin of the safflower is supposed to be the Northern Africa or Near East Asia (Valvilov, 1950). De Candolla (1885) felt that Arabia was more probable, but more recent work by Hanelt (1963) on taxonomy, Ashri (1974) on the divergence and evolution of the genus, and Ashri & Knowles (1960) on cytogenetics lead to the conclusion that Euphrates basin is the most likely area for its origin. Though the number of individuals sampled for analysis was small and probably not fully representatives of the total available diversity in *C. tinctorius*, our study demonstrates that the regions (Turkey and Egypt) of the Mediterranean Sea are more variable than other regions (Table 3). In this result, although only simple result of ISSR is difficult to assert the center of species diversity of *C. tinctorius*, the regions of the Mediterranean Sea may be the most probable candidate for the origin of safflower. India was also the candidate of the center or secondary center of species diversity of *C. tinctorius*.

The possibility of quickly obtaining polymorphism for genetic mapping or fingerprinting was tested by designing two repeated primers of $(CA)_{10}$, $(AT)_{10}$, $(CAT)_5$, and $(CAT)_6$ (Table 2). Two repeated primers were more amplified bands for safflower than three repeated primers. It was similar to result that the proper oligonucleotide fingerprinting proved to be a useful tool to analyze genetic diversity in Lima bean (Lioi & Galasso, 2002).

Within the major clade, associated between geographic distance and genetic divergence were somewhat more tenuous (Fig. 3). For example Morocco (MAR) is not clustered with the African clade (Egypt and Ethiopia) and the African clade was sistered Azerbaijan (AZE). Cultivated varieties of *C. tinctorius* have diverged because of cultivation by man in semi-isolated regions of the Mediterranean. Geographic areas with distinct centers of similarity for cultivated safflower, and the generalized characteristics of these varieties have been described (Knowles 1969; Ashri *et al.*, 1975). Both Knowles (1969) and Ashri *et al.* (1975) noted that these groupings were based on generalizations and that gene flow by introductions of cultivars from different regions was ongoing and prevents complete divergence.

Conclusion based on the results study, ISSR markers were the least polymorphic markers of those evaluated and consequently had the least resolving power.

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