RESEARCH

Molecular and Morphological Variation in a World-Wide Collection of Safflower

Mohammad Mahdi Majidi* and Soheila Zadhoush

ABSTRACT

Safflower (*Carthamus tinctorius* L.) is one of the oldest domesticated crops, mainly grown as oilseed in the arid and semiarid regions of the world. Wild relatives of safflower comprise several species distributed worldwide. This study was conducted to investigate the genetic diversity of safflower germplasm and to identify the relationships among some *Carthamus* species using intersimple sequence repeats (ISSR) and morphological characteristics. Genetic materials consisted of 102 accessions (from 46 different countries) from seven species of *Carthamus*, including cultivated *C. tinctorius* and wild species of *C. lanatus*, *C. oxyacanthus*, *C. glaucus*, *C. boissieri*, *C. dentatus*, and *C. palaestinus*. High levels of variation were found for morphological traits; especially seed yield and number of seeds per head. Morphological assessment and clustering could not completely categorize accessions with the related species. Molecular analysis revealed a total of 508 ISSR marker loci, of which 72% were polymorphic with a broad range of 14 to 25 amplified products per primer. Polymorphic information content values ranged from 0.19 to 0.45, with an average of 0.36. Cluster analysis and principal coordinate analysis (PCA) based on molecular data clearly separated accessions according to their species. As expected, *C. palaestinus* genotype were grouped together with genotypes of *C. tinctorius*. All genotypes of cultivated species were clustered into five subgroups, mainly based on their geographical distributions. The similarity coefficient between species ranged from 0.34 (between *C. tinctorius* and *C. lanatus*) to 0.90 (between *C. tinctorius* and *C. palaestinus*). The relationship among the regional gene pools of safflower was also discussed.

M.M. Majidi, Dep. of Agronomy and Plant Breeding, and S. Zadhoush, Dep. of Agronomy and Plant Breeding, College of Agriculture, Isfahan Univ. of Technology, 84156-8311, Iran. Received 23 Dec. 2013. *Corresponding author (majidi@cc.iut.ac.ir).

Abbreviations: AMOVA, analysis of molecular variation; GCV, genotypic coefficients of variation; He, Nei's genetic diversity; I, Shanon's information index; ISSR, intersimple sequence repeats; P, percentage of polymorphic loci; PCA, principal coordinate analysis; PCR, polymerase chain reaction; PCV, phenotypic coefficients of variation; PIC, polymorphism information content; RAPD, randomly amplified polymorphic DNA; Rp, resolving power.

Safflower is one of the oldest oilseed crops traditionally grown for its flowers, which are largely used for dyes, teas, and food additives. It is also cultivated to produce a type of cooking oil rich in polyunsaturated fatty acids, for animal and bird food (Mundel and Bergman, 2008). It is believed to have been domesticated somewhere in the Fertile Crescent region over 4000 yr ago (Ashri, 1975; Knowles, 1969). World production of safflower is lower than some oilseed crops due to its relatively lower seed yield and a series of biotic and abiotic stresses (Ashri, 1975). Therefore, it is necessary to identify different gene sources to create resistance to various stresses through breeding programs. Wild species of safflower are an important reservoir of useful genes and can be exploited both to broaden the existing genetic base and to enrich the existing varieties with agronomically favorable traits. Genetic analysis of crop gene pools and recognition of phylogenetic relationships can provide insight into the origin and subsequent evolution of crop plants and can also result in the identification of

Published in Crop Sci. 54:2109–2119 (2014).
doi: 10.2135/cropsci2013.12.0850

novel sources of genetic variation for continued improvement of crop plants (Chapman et al., 2010).

The genus *Carthamus* consists of 15 to 25 species distributed worldwide (Bowles et al., 2010; Kumar, 1991; Vilatersana et al., 2005). To classify the genus *Carthamus*, researchers have benefited from morphological, cytogenetic, anatomical, and molecular studies (Chapman and Burke, 2007; Sehgal et al., 2008). Safflower has four categories of chromosome numbers including $2n$ = 20, 24, 44, and 64 (Ashri and Knowles, 1960). Based on the four classes of chromosome numbers, the genus was categorized into four sections. For instance, according to Ashri and Knowles (1960), Section 1 ($2n$ = 24) includes the annual species *C. tinctorius*, *C. palaestinus*, *and C. oxyacanthus*. All of these three species cross readily and produce fertile hybrids (Mayerhofer et al., 2010). Both *C. palaestinus and C. oxyacanthus* have been implicated as the wild progenitor of *C. tinctorius* (Ashri and Knowles, 1960; Chapman and Burke, 2007). Section 2 ($2n$ = 20) contains *C. alexandrines*, *C. glaucus*, *C. boissieri*, and *C. dentatus*. Section 3 ($2n$ = 44) consists of only one species (*C. lanatus*). Crossing of *C. lanatus* with species of section 1 showed low pairing between chromosomes. Finally, Section 4 ($2n$ = 64) comprises two species, namely *C. baeticus* and *C. turkestanicus*. Studying the DNA of chloroplasts showed that *C. palaestinus* and *C. oxycanthus* are the wild ancestors of cultivated safflower (Sehgal et al., 2008). However, sequencing of seven genes in the phylogenetic study revealed a closer relationship between cultivated safflower and *C. palaestinus* (Chapman and Burke, 2007). While phylogenetic relationships between species of safflower have also been evaluated by some molecular markers such as randomly amplified polymorphic DNA (RAPD), expressed sequence tag–simple sequence repeat, and sequences nuclear genes (Vilatersana et al., 2005; Sasanuma et al., 2008; Chapman et al., 2009), the ISSR technique has not been used for this purpose.

Understanding genetic diversity in germplasm collections can greatly facilitate reliable classification of accessions and identification of subsets of core accessions with possible utility in breeding programs (Mohammadi and Prasana, 2003). Different methods such as assessment of morphological, anatomical, and molecular characteristics have been employed to investigate genetic diversity. Morphological characteristics have a number of limitations like low polymorphism and heritability and are influenced by stage of plant development. In contrast to morphological and anatomical traits, molecular markers detect diversity at the DNA level and largely overcome the problems associated with morphological-based classification (Awasthi et al., 2004). The ISSR markers give multilocus patterns that are very reproducible, abundant, highly polymorphic in plant genomes, extremely informative, and quick to use (Zietkiewicz et al., 1994). Comparisons of ISSR markers with other polymerase chain reaction (PCR)-based markers has revealed their efficiency for DNA fingerprinting and assessment of genetic diversity in plant breeding (Zietkiewicz et al., 1994; Archak et al., 2003; Galvan et al., 2003; Huang et al., 2010; Reunova et al., 2010; Smolik et al., 2010).

Knowles (1969) introduced the Far East (China, Japan, and Korea), India–Pakistan, the Middle East, Egypt, Sudan, Ethiopia, and Europe as seven diversity centers for safflower. Ashri (1975) extended the number of centers to 10 and called them "regional gene pools." The Middle East center was subdivided into three gene pools (Iran–Afghanistan, Israel–Jordan–Iraq–Syria, and Turkey) with the Kenya gene pool added to the list (Ashri, 1975). The cultivated species of safflower is the only species of this genus, mainly grown as an oilseed crop in arid and semiarid regions of the world. However, little information is available about the genetic diversity and the relationship among its regional gene pools. The present study aimed (i) to investigate genetic diversity of safflower genotypes from different regions of the world using ISSR markers and morphological traits, (ii) to determine phylogenetic relationships between some important species of the genus *Carthamus*, and (iii) to assess the ability of the ISSR marker system to determine the relationship between safflower regional gene pools.

MATERIALS AND METHODS

Plant Material

One hundred and two accessions of seven species of the genus *Carthamus* from 46 different countries were used in this study (Table 1). These accessions included 79 genotypes of cultivated species (*C. tinctorius*), nine genotypes of *C. lanatus*, seven genotypes of *C. oxyacanthus*, four genotypes of *C. glaucus*, and one genotype from each of the species *C. boissieri*, *C. dentatus*, and *C. palaestinus*. Iranian accessions were collected from different geographical regions nationwide. Exotic accessions were kindly provided by the gene bank of the Leibniz Institute of Plant Genetics and Crop Plant Research and the United States Department of Agriculture (USDA). Most of the 79 genotypes of cultivated species (*C. tinctorius*) belonged to centers of similarity or regional gene pools (Knowles, 1969; Ashri, 1975). The remaining accessions were obtained from Australia, Canada, Mexico, and the United States.

Morphological Analysis

Accessions were evaluated in the field during two growing seasons (2010–2011, 2011–2012). At each growing season, the experiment was conducted according to a randomized complete block design with three replications. Sowing was done by hand in plots consisting of two rows, 3 m in length and 30 cm apart. The plants were spaced 10 cm apart within rows. The experiment was performed on a Typic Haplargid, silty clay loam soil at Isfahan University of Technology Research Farm (32°30′ N, 51°20′ E, Isfahan, Iran). The soil was calcareous, containing 370 g/kg calcium carbonate equivalent, 6.0 g/kg organic C, and 0.77 g/kg total N (pH = 8.1). The soil was nonsaline and nonsodic. The mean annual temperature and precipitation were 14.5°C and 140 mm, respectively. Irrigation supply was nonlimited and

Table 1. Information of safflower germplasm investigated in this study.

No.	PI No.	Species	Geographical location	Molecular cluster	Morphological cluster
1	PI 198843	*C. tinctorius*	France	A_I	A_I
2	PI 198844	*C. tinctorius*	France	A_I	A_I
3	PI 209286	*C. tinctorius*	Romania	A_I	A_I
4	PI 209287	*C. tinctorius*	Romania	A_I	A_I
5	PI 209299	*C. tinctorius*	Kenya	A_I	A_I
6	PI 209300	*C. tinctorius*	Kenya	A_I	A_I
7	PI 239707	*C. tinctorius*	Turkey	A_{III}	A_I
8	PI 239708	*C. tinctorius*	Turkey	A_{III}	A_I
9	PI 253516	*C. tinctorius*	Germany	A_I	A_I
10	PI 576991	*C. tinctorius*	Germany	A_I	A_I
11	PI 253519	*C. tinctorius*	Austria	A_I	A_I
12	PI 253520	*C. tinctorius*	Austria	A_I	A_I
13	PI 253521	*C. tinctorius*	Italy	A_I	A_I
14	PI 253522	*C. tinctorius*	Italy	A_I	A_I
15	PI 253541	*C. tinctorius*	Hungary	A_I	A_I
16	PI 253544	*C. tinctorius*	Poland	A_I	A_I
17	PI 311737	*C. tinctorius*	Poland	A_I	A_I
18	PI 253548	*C. tinctorius*	Denmark	A_I	A_I
19	PI 253560	*C. tinctorius*	Morocco	A_I	A_I
20	PI 253561	*C. tinctorius*	Switzerland	A_I	A_I
21	PI 253759	*C. tinctorius*	Iraq	A_{III}	A_I
22	PI 253762	*C. tinctorius*	Iraq	A_{III}	A_I
23	PI 254976	*C. tinctorius*	Greece	A_I	A_I
24	PI 262424	*C. tinctorius*	Australia	A_{II}	A_I
25	PI 262425	*C. tinctorius*	Australia	A_{II}	A_I
26	PI 279343	*C. tinctorius*	Japan	A_V	A_I
27	PI 286199	*C. tinctorius*	Kuwait	A_{III}	A_I
28	PI 286385	*C. tinctorius*	Eritrea	A_I	A_I
29	PI 286386	*C. tinctorius*	Eritrea	A_I	A_I
30	PI 305527	*C. tinctorius*	Sudan	A_{II}	A_I
31	PI 305528	*C. tinctorius*	Sudan	A_{II}	A_I
32	PI 306684	*C. tinctorius*	Israel	A_{III}	A_I
33	PI 369843	*C. tinctorius*	Uzbekistan	A_I	A_I
34	PI 369844	*C. tinctorius*	Uzbekistan	A_{III}	A_I
35	PI 369853	*C. tinctorius*	Uzbekistan	A_V	A_I
36	PI 369854	*C. tinctorius*	Uzbekistan	A_{III}	A_I
37	PI 369845	*C. tinctorius*	Tajikistan	A_{III}	A_I
38	PI 369847	*C. tinctorius*	Tajikistan	A_{III}	A_I
39	PI 369848	*C. tinctorius*	Ukraine	A_I	A_I
40	PI 386173	*C. tinctorius*	Syria	A_{III}	A_I
41	PI 386174	*C. tinctorius*	Syria	A_{III}	A_I
42	PI 401470	*C. tinctorius*	Bangladesh	A_{IV}	A_I
43	PI 470942	*C. tinctorius*	Bangladesh	A_{III}	A_I
44	PI 426188	*C. tinctorius*	Afghanistan	A_{III}	A_I
45	PI 426189	*C. tinctorius*	Afghanistan	A_{IV}	A_I
46	PI 532619	*C. tinctorius*	Cyprus	A_I	A_I
47	PI 537652	*C. tinctorius*	Mexico	A_I	A_I
48	PI 657789	*C. tinctorius*	Mexico	A_I	A_I
49	PI 657790	*C. tinctorius*	Mexico	A_I	A_I
50	PI 572425	*C. tinctorius*	United States	A_{III}	A_I
51	PI 572426	*C. tinctorius*	United States	A_{III}	A_I
52	PI 653202	*C. tinctorius*	India	A_{III}	A_I
53	PI 657787	*C. tinctorius*	India	A_{IV}	A_I
54	PI 653213	*C. tinctorius*	China	A_V	A_I
55	PI 657817	*C. tinctorius*	China	A_V	A_I
56	PI 657800	*C. tinctorius*	Egypt	A_{II}	A_I
57	PI 657801	*C. tinctorius*	Egypt	A_{II}	A_I
58	PI 657819	*C. tinctorius*	Jordan	A_{III}	A_I
59	PI 657820	*C. tinctorius*	Jordan	A_{III}	A_I
60	PI 657823	*C. tinctorius*	Palestinian Territory	A_{IV}	A_I
61	CART 64	*C. tinctorius*	Slovakia	A_I	A_I
62	CART 32	*C. tinctorius*	Germany	A_I	A_I
63	CART 87	*C. tinctorius*	Romania	A_I	A_I
64	CART 56	*C. tinctorius*	United States	A_{III}	A_I
65	CART 124	*C. tinctorius*	Pakistan	A_{IV}	A_I
66	CART 70	*C. tinctorius*	Libya	A_I	A_I
67	CART 126	*C. tinctorius*	Belgium	A_I	A_I
68	CART 55	*C. tinctorius*	Poland	A_I	A_I
69	CART 78	*C. tinctorius*	Hungary	A_I	A_I
70	CART 103	*C. tinctorius*	Canada	A_{III}	A_I
71	CART 79	*C. tinctorius*	Japan	A_V	A_I
72	CART 50	*C. tinctorius*	Bulgaria	A_I	A_I
73	–	*C. tinctorius*	Iran	A_{III}	A_I
74	–	*C. tinctorius*	Iran	A_{III}	A_I
75	–	*C. tinctorius*	Iran	A_{III}	A_I
76	–	*C. tinctorius*	Iran	A_{III}	A_I
77	–	*C. tinctorius*	Iran	A_{III}	A_I
78	–	*C. tinctorius*	Iran	A_{III}	A_I
79	–	*C. tinctorius*	Iran	A_{III}	A_I
80	CART 91	*C. boissieri*	Cyprus	D	B
81	PI 235663	*C. palaestinus*	Israel	A_{III}	A_I
82	PI 235668	*C. lanatus*	Netherlands	C	C
83	W616791	*C. lanatus*	China	C	C
84	–	*C. lanatus*	Iran	C	C
85	CART 46	*C. lanatus*	France	C	C
86	CART 82	*C. lanatus*	Georgia	C	C
87	CART 53	*C. lanatus*	Tunisia	C	C
88	CART 84	*C. lanatus*	Kyrgyzstan	C	C
89	CART 71	*C. lanatus*	Afghanistan	C	C
90	PI 426425	*C. lanatus*	Afghanistan	C	C
91	–	*C. oxyacanthus*	Iran	B	D
92	–	*C. oxyacanthus*	Iran	B	D
93	–	*C. oxyacanthus*	Iran	B	D
94	–	*C. oxyacanthus*	Iran	B	D
95	PI 426185	*C. oxyacanthus*	Afghanistan	B	D
96	PI 426467	*C. oxyacanthus*	Pakistan	B	D
97	PI 426470	*C. oxyacanthus*	Pakistan	B	D
98	CART 20	*C. glaucus*	–	E	B
99	CART 52	*C. glaucus*	–	E	B
100	CART 45	*C. glaucus*	Israel	E	B
101	CART 92	*C. glaucus*	Greece	E	B
102	CART 88	*C. dentatus*	–	F	B

Table 2. Means of agromorphological traits in different accessions of four species of *Carthamus*.

Trait	*C. glaucus*	*C. lanatus*	*C. oxyacanthus*	*C. tinctorius*
Days to shooting	65.6 ± 2	76.9 ± 11	61.4 ± 4	49 ± 2.6
Days to heading	83.8 ± 4.1	98.8 ± 9.4	81.6 ± 2.2	76 ± 2
Days to flowering	119 ± 2	122.2 ± 1	102.2 ± 3.8	96 ± 2
Days to maturity	152.1 ± 4.8	154 ± 8	140.4 ± 5.1	128.7 ± 4
Seed yield, g plant^{-1}	13.5 ± 4.3	8.7 ± 6.2	12.4 ± 3.8	27.5 ± 4.7
Thousand-seed weight	26.1 ± 12.6	27.7 ± 12.2	18.6 ± 3.6	33.7 ± 2.3
Seed and head weight, g plant^{-1}	34 ± 11.3	28.5 ± 17.8	44.8 ± 11.1	54.5 ± 8.7
No. of heads per plant	30.6 ± 1	28.9 ± 15.3	60.7 ± 45.6	23.1 ± 2.8
Heads diameter, cm	2.9 ± 4.1	1.5 ± 0.5	1.5 ± 3.1	2.4 ± 2.1
No. of branches	11.8 ± 11	6.8 ± 3.3	12.0 ± 6.4	8.6 ± 1
Plant height, cm	81.4 ± 12	70.6 ± 31.1	79.8 ± 15.8	96.6 ± 6.4
No. of seeds per head	15.4 ± 2.78	13.4 ± 2.56	17.36 ± 3.82	29.89 ± 4.20

performed when 50% of the total available water was depleted from the root zone during the growing season. Twelve morphological traits, including days to shooting, days to heading, days to flowering, days to maturity, seed yield (g/plant), thousand-seed weight, seed and head weight, number of heads per plant, head diameter (cm), plant height (cm), number of branches, and number of seeds per head, were measured for each accession (Table 2). For each plot, the measurements were taken on 20 plants and the mean value was used for subsequent analysis.

Intersimple Sequence Repeat Analysis

Seeds were placed on moist filter paper to break dormancy and facilitate germination. Seedlings were then transferred to soil and allowed to grow for 6 wk before DNA extraction. Young leaves of 15 plants from each genotype were harvested, pooled and stored at −80°C. Total DNA was extracted following the Murray and Thomson (1980) methods. Of the 50 ISSR primers screened, 20 produced a higher number of reproducible bands and were selected for ISSR analysis (Table 4). The PCR analysis was performed in a 15 μL total volume solution containing 20 ng total DNA, 1.5 10× PCR buffer, 2 m*M* $MgCl_2$, 0.3 m*M* dNTP, 5 p*M* of each primer, and 0.5 U Taq DNA polymerase. Amplification was done in a thermocycler (Bio-Rad Laboratories, Hercules, CA) with initial denaturation at 94°C for 2 min, 40 cycles of 94°C for 1 min, specific angling temperature for 1 min, 72°C for 2 min, and final extension step at 72°C for 5 min. The amplified DNA fragments were separated in a 1.5% agarose gel at 100 W for 2 h in 1× TBE buffer (100 m*M* Tris-Borate, pH 8.0, 2 m*M* EDTA) and stained with ethidium bromide.

Data Analysis

Analysis of variance for morphological traits was performed using SAS statistical software (SAS Institute, 1999). Cluster analysis (based on Euclidean dissimilarity matrix and ward method) was performed after data standardization (Z score) using Statistical Package for Social Science for Windows 19.0 (SPSS Inc., Chicago, IL). Phenotypic and genotypic coefficients of variation (PCV and GCV, respectively) were calculated using the following formulas:

$$PCV = (\sigma_p/\mu)\ 100$$

$$GCV = (\sigma_g/\mu)\ 100$$

where σ_p, σ_g, and μ are phenotypic standard deviation, genotypic standard deviation, and mean of the traits, respectively.

For molecular analysis, only sharp and precise bands were scored as 1 (present) and 0 (absent) in the data matrix. The polymorphism information content (PIC) and resolving power (Rp) of each ISSR marker was computed using the following formulas:

$$PIC_i = [2f_i \times (1 - f_i)]$$

$$Rp_i = \Sigma[1 - (2 \times |0.5 - f_i|)]$$

(Roldán-Ruiz et al., 2000, and Prevost and Wilkinson, 1999, respectively) where *i* represents the *i*th primer, f_i is the frequency of the amplified allele, and $(1 - f_i)$ is the frequency of the null allele of the *i*th primer. Genetic similarity among all accessions was calculated according to the Jaccard (**J**) similarity index (Mohammadi and Prasana, 2003). Cluster analysis (complete linkage method) and PCA were then performed using NTSYSpc 2.02 (Rohlf, 1998). The classifications made by morphological and ISSR data were compared with the Mantel test (Mantel, 1967). The cophenetic correlation coefficient was generated by means of the COPH routine to check the goodness of fit between the clusters in the dendrogram and the similarity coefficient matrix.

The accessions of *C. tinctorius* were divided into 10 regional gene pools, and the mean genetic similarity was calculated between them. Besides, all accessions of *C. tinctorius* were categorized into six centers of similarity according to Knowles (1969). Afterward, Nei and Li's index (H), Nei's genetic diversity (He), Shanon's information index (I), and the percentage of polymorphic loci (P) were estimated using Popgen software 1.32 (Yeh et al., 1999). Analysis of molecular variation (AMOVA) among the mentioned groups was performed in Arliquin software 3.0 (Excoffier et al., 2005).

Table 3. Means, range, phenotypic coefficients of variation (PCV), and genotypic coefficients of variation (GCV) of agromorphological traits in 79 accessions of cultivated safflower.

Trait	Mean	Range (Min.–Max.)	PCV	GCV
Days to shooting	45	43–49	9.22	8.01
Days to heading	76	74–78	12.45	11.03
Days to flowering	96	93–99	16.36	14.58
Days to maturity	128.7	125–135	25.64	23.79
Seed yield, g plant^{-1}	27.45	19.5–34.7	80.52	68.41
Thousand-seed weight	37.7	29.1–48	11.33	10.93
Seed and head weight, g plant^{-1}	54.5	41.5–74.7	50.89	46.77
No. of heads per plant	23.1	19–29	25.32	22.32
Heads diameter, cm	2.4	2.2–2.7	26.83	23.89
No. of branches	8.6	7–10	30.21	28.07
Plant height, cm	96.6	88–109	46.82	40.83
No. of seeds per head	35.6	28–42	75.17	68.15

RESULTS

Agromorphological Study

According to the results of ANOVA, there was a large and significant difference ($P < 0.01$) between accessions for all of the measured traits (data not shown), which indicated high levels of variation in the studied germplasm. The mean values of agromorphological traits in four Carthamus species (with more than one accession) are presented in Table 2. Accessions of *C. tinctorius* had the highest seed yield (27.5 g/plant) and the fewest days to flowering (96 d). On the other hand, *C. lanatus* had the lowest seed yield (8.7 g/plant) and the longest days to flowering (122 d; Table 2). The mean value, range, and GCV were calculated for 12 agromorphological traits and presented in Table 3. Seed yield (68.4%) and number of seeds per head (68.1%) had the highest GCV and showed the highest variation among the morphological traits. The lowest variability belonged to days to shooting (8.0%), thousand-seed weight (10.9%), and days to heading (11.0%; Table 3).

The result of clustering based on morphological distance matrix of 102 accessions was shown in Table 1. On this basis, the accessions were grouped in four clusters (A, B, C, and D). Cluster A contained all the accessions of *C. tinctorius* and *C. palestinus*. Morphological assessment could not distinguish between the accessions of the three species *C. glaucus*, *C. dentatus*, and *C. boissieri*, which were all grouped in Cluster B. All accessions of *C. lanatus* were categorized in a separate cluster (Cluster C) and accessions of *C. oxyacanthus* were grouped in Cluster D.

Molecular Study

A total of 508 amplification products were produced by 20 ISSR primers. Of these, 368 (72.4%) were polymorphic across the 102 accessions (Table 4). The average frequency of polymorphic bands was 18.4 per primer. The amplified DNA ranged from 300 bp to 2000 bp in size (Table 4).

Table 4. Percentage of polymorphism (P), polymorphic information content (PIC), and resolving power (Rp) values for polymorphic primers of intersimple sequence repeats used in different accessions of safflower.

No.	Primer sequence (5′–3′)	Total No. of bands	No. of polymorphic bands	P	PIC	Rp
1	$(GAA)_6$	25	20	80	0.34	7.8
2	$(GACA)_5$	28	20	71	0.39	8.7
3	$(GA)_8RC$	19	15	78.9	0.39	5.63
4	$(CA)_8GT$	23	18	78.2	0.36	11.82
5	$(GT)_8YC$	31	25	80.6	0.34	13.45
6	$(TG)_8G$	29	19	65.5	0.45	8.5
7	$(AC)_8YG$	32	25	78.1	0.40	13.8
8	$(AG)_8T$	28	20	71.4	0.44	11.22
9	$(CA)_8RT$	19	15	78.9	0.44	13.68
10	$(CA)_8WT$	25	18	72	0.42	9.82
11	$(GA)_8C$	24	19	79.1	0.33	6.2
12	$(CT)_8A$	30	22	73.3	0.24	5.25
13	$(CT)_8G$	26	18	69.2	0.38	7.35
14	$(AC)_8T$	25	17	68	0.41	4.33
15	$(GA)_8YT$	22	17	77.2	0.40	8.25
16	$(GA)_8YC$	25	17	68	0.31	6.02
17	$(AG)_8YT$	24	17	70.8	0.40	11.20
18	$(Ct)_8RC$	23	18	78.2	0.43	6.25
19	$(GAGA)_4$	24	14	58.3	0.22	9.87
20	$(CT)_8RA$	26	14	53.8	0.19	5.30
Total	–	508	368	–	–	174.4
Mean	–	25.4	18.4	72.4	0.36	8.72

The percentage of polymorphic bands ranged from 53.8 to 80.6% for primers $(CT)_8RA$ and $(GT)_8YC$, respectively. The PIC values ranged from 0.19% [$(CT)_8RA$] to 0.45% [$(TG)_8G$] with an average of 0.36. The maximum and minimum Rp belonged to $(CT)_8A$ and $(CA)_8RT$, respectively (Table 4). Jaccard similarity coefficient between safflower accessions ranged from 0.19 (PI 426189 and CART 50) to 0.98 (PI 239707 and PI 239708) with an average of 0.53 (data not shown).

The complete linkage cluster analysis based on the Jaccard similarity matrix for ISSR data is summarized in Fig. 1 and Table 1. All 102 accessions were divided into six main groups. Except for *C. tinctorius* and *C. palaestinus*, ISSR analysis could categorize accessions of the studied species into separate groups. This grouping was confirmed by PCA (Fig. 2). Cluster A contained 79 accessions of *C. tinctorius* and the probable wild progenitor of safflower, *C. palaestinus*. Moreover, accessions of this cluster were further classified to five subclusters generally based on their geographical distributions (Fig. 1). Subcluster A_I included 34 accessions, mainly from the regional gene pool of Europe, along with accessions from Mexico, Kenya, Morocco, and Libya. Subcluster A_{II} comprised six accessions from the regional gene pool of Egypt and Sudan, along with accessions from Australia. Subcluster A_{III} consisted of 29 accessions from the Middle East, Iran, Afghanistan, Canada,

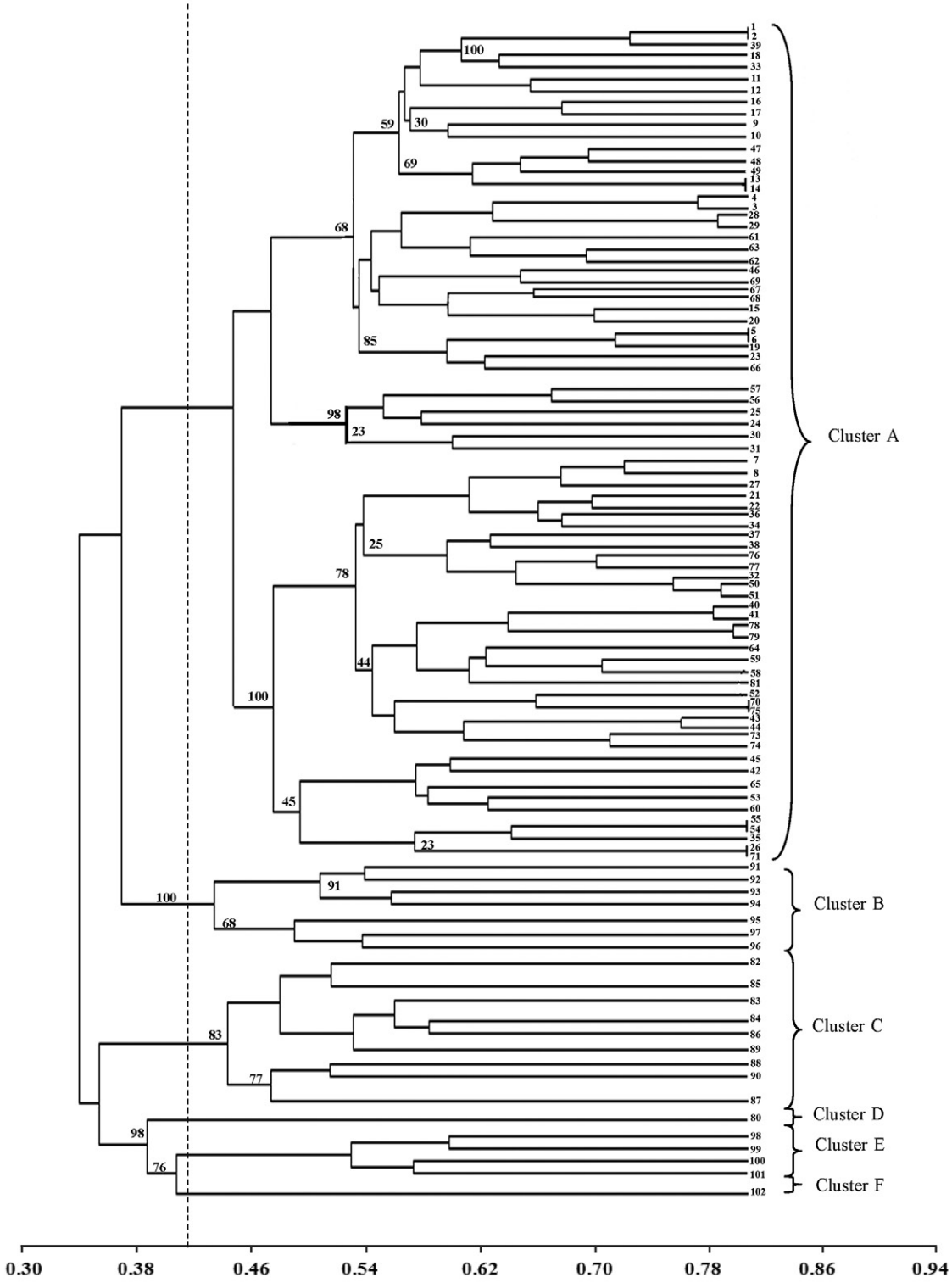

Figure 1. Unweighted pair group method with arithmetic mean dendrogram of 102 accessions of seven *Carthamus* species based on intersimple sequence repeat markers data. For explanation of details of species and origin of the accessions see Table 1.

and the United States. Subcluster A_{IV} was comprised of five accessions from Afghanistan, Bangladesh, Pakistan, and Israel. Finally, Subcluster A_V consisted of five accessions from China, Japan, and Uzbekistan. Clusters B, C, D, E, and F were comprised of accessions from *C. oxyacanthus*, *C. lanatus*, *C. boissieri*, *C. galucus*, and *C. dentatus*, respectively.

Nei's genetic similarity and distance coefficient values among safflower species are calculated according to Yeh et al. (1999) and presented in Table 5. The similarity coefficient between species ranged from 0.34 (between *C. tinctorius* and *C. lanatus*) to 0.90 (between *C. tinctorius* and *C. palaestinus*). The similarity coefficient between *C.*

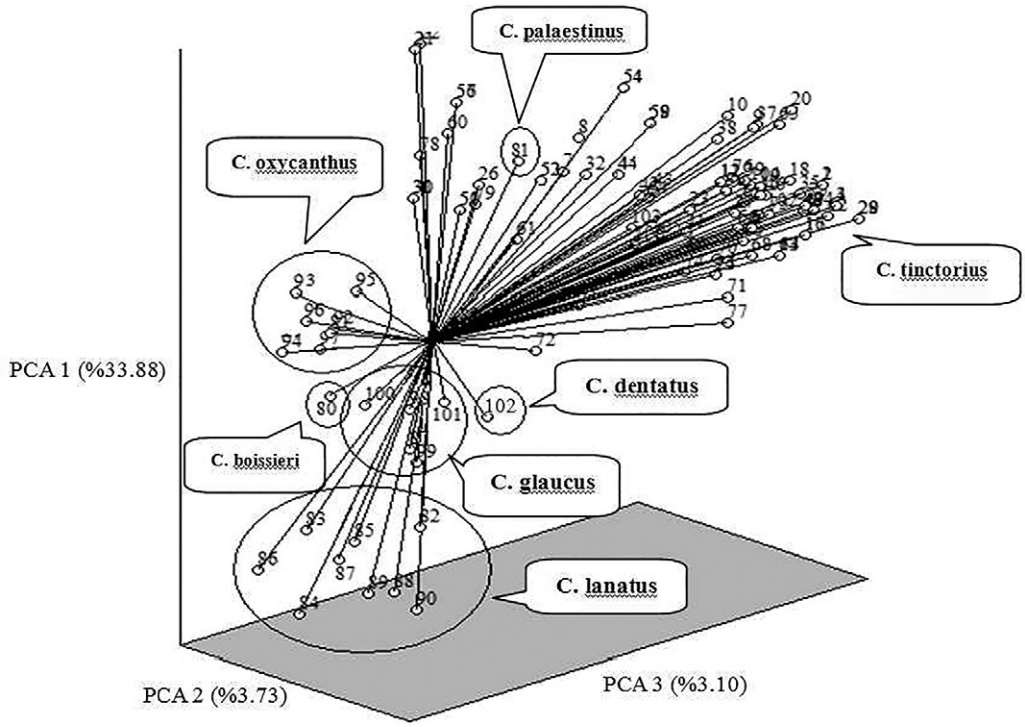

Figure 2. Genetic relationships among 102 accessions belonged to seven species of *Carthamus* by principle coordinate analysis (PCA). Numbers on the each circle correspond to the genotypic number listed in Table 1.

Table 5. Mean genetic similarity (above diagonal) and genetic distance (below diagonal) between seven species of *Carthamus* by Nei's method.

Species	1	2	3	4	5	6	7
1- *C. tinctorius*		0.727	0.903	0.348	0.852	0.45	0.395
2- *C. boissieri*	0.318		0.413	0.485	0.597	0.794	0.680
3- *C. palaestinus*	0.096	0.586		0.381	0.736	0.630	0.653
4- *C. lanatus*	0.651	0.514	0.618		0.550	0.438	0.557
5- *C. oxycanthus*	0.147	0.402	0.263	0.449		0.876	0.797
6- *C. glaucus*	0.549	0.235	0.461	0.561	0.132		0.774
7- *C. dentatus*	0.604	0.320	0.425	0.442	0.225	0.255	

palaestinus and *C. oxyacanthus* was 0.73. An AMOVA was performed to differentiate the four species (with more than one species) and to estimate the percentage of intra- and interspecies genetic variation (data not shown). Significant variation was observed among the species ($F = 0.12$; $P < 0.0001$). The results of AMOVA revealed that 87.2% of the total genetic variation occurred within species and only 12.8% was seen between species.

Estimates of genetic similarities between 10 regional gene pools for 79 accessions of cultivated safflower using ISSR markers (Table 6) showed the least similarity between Sudan and Australia (0.54). The maximum similarity was detected between the Middle East and Iran–Afghanistan (0.93) and also between the Middle East and Europe (0.93). A dendrogram of 10 regional gene pools based on the ISSR data on 79 worldwide accessions of *C. tinctorius* is illustrated in Fig. 3. As it is seen, Australia, Egypt, and Sudan were categorized in the same cluster. In addition, the gene pools of Europe, Africa, and the Americas (Canada, United States, and Central and South America) had the greatest similarity.

Diversity statistics were estimated to compare levels of genetic diversity between the six centers of similarity of cultivated safflower (Table 7). According to the obtained results, among the similarity centers, the Middle East (H = 0.31; I = 0.47; P = 94%) and the Far East (H = 0.16; I = 0.24; P = 47%) had the highest and lowest levels of genetic variability, respectively. Again, AMOVA was applied to differentiate between the six centers of similarity of *C. tinctorius* and to estimate the percentage of intra- and intercenter genetic variation (data not shown). A significant variation was observed between the studied centers ($F = 0.14$; $P < 0.0001$). The AMOVA also indicated that 77.9% of total genetic variation occurred within the centers and only 22.1% was related to variations between the centers.

The cophenetic correlation coefficient (*r* value, a measure to check the goodness of fit of a cluster analysis to the associated similarity matrix) for ISSR data was 0.92,

Table 6. Mean genetic similarity (above diagonal) and genetic distance (below diagonal) between 10 regional gene pool of *Carthamus tinctorius* by Nei's method.

Gene pool	Europe	Africa	Middle East	Australia	Far East	India-Pakistan	Iran-Afghanistan	United States	Egypt	Sudan
Europe		0.91	0.93	0.79	0.81	0.86	0.91	0.90	0.73	0.68
Africa	0.08		0.85	0.77	0.74	0.79	0.85	0.85	0.63	0.60
Middle East	0.06	0.15		0.74	0.86	0.91	0.93	0.88	0.79	0.74
Australia	0.23	0.25	0.29		0.67	0.74	0.74	0.77	0.63	0.54
Far East	0.20	0.28	0.14	0.38		0.82	0.85	0.81	0.68	0.74
India-Pakistan	0.14	0.22	0.08	0.29	0.18		0.87	0.85	0.76	0.68
Iran-Afghanistan	0.09	0.16	0.06	0.29	0.15	0.13		0.88	0.80	0.70
United States	0.10	0.15	0.12	0.25	0.20	0.16	0.12		0.74	0.66
Egypt	0.30	0.44	0.22	0.45	0.37	0.27	0.21	0.28		0.60
Sudan	0.37	0.45	0.28	0.60	0.30	0.37	0.34	0.40	0.52	

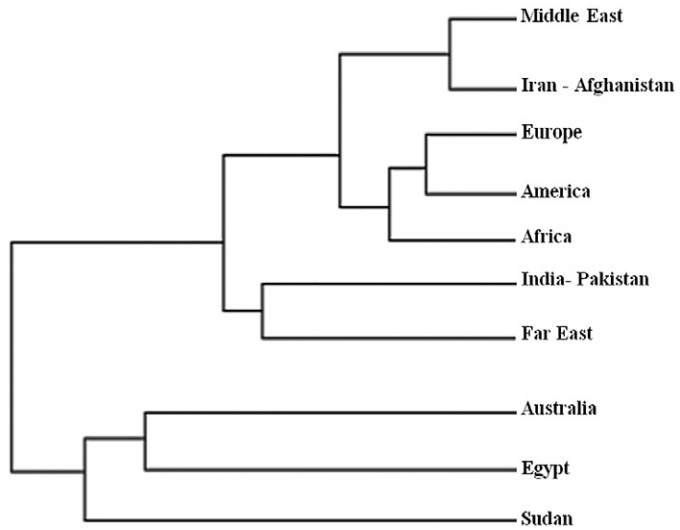

Figure 3. Dendrogram of 10 regional gene pools based on the intersimple sequence repeat data on 79 worldwide accessions of *Carthamus tinctorius*.

Table 7. Percentage of polymorphic loci (P), Nei's genetic diversity (He), and Shanon's information index (I) values for six center of similarity of *Carthamus tinctorius*.

Center of similarity	P	I	He
Europe	82.67	0.43	0.29
Africa	72.67	0.42	0.28
Middle East	94	0.47	0.31
Far East	46.67	0.24	0.16
Iran–Afghanistan	78.67	0.40	0.27
India–Pakistan	52.67	0.26	0.17
Mean	71.22	0.37	0.25

suggesting a very good fit between the dendrogram and the corresponding similarity matrices from which it was derived. The Mantel matrix test, however, showed poor correlation (r = 0.4; P = 0.01) between the similarity matrices obtained with the ISSR and morphological data.

DISCUSSION

Breeding programs of oilseed crops require information about the level of the genetic variation of different characteristics and genetic relationships of related species for planning intraspecific hybridization. Genetic studies on safflower have been slow, and limited knowledge is thus available on the level of genetic diversity of its germplasm. To the best of our knowledge, the present study represented the first attempt to investigate a broad spectrum of variation in safflower germplasm at both morphological and DNA levels. It also assessed the relationship of wild relatives of safflower using morphological and ISSR markers.

Morphological Analysis

We found a wide range of genetic diversity in agromorphological traits among the studied safflower germplasm. Morphological assessment could only distinguish the studied species according to their sections defined by Ashri and Knowles (1960). Morphological traits are commonly used to analyze genetic diversity and provide a simple way of quantifying genetic variation (Fufa et al., 2005). However, limitations such as low polymorphism and heritability (environmental effects; Amini et al., 2008) may obscure true differences. Molecular markers may overcome the problems associated with morphological classifications.

Higher seed yield of cultivated safflower compared with the wild species suggested selection intensity to improve its productivity during domestication process. However, wild species may be a good source of genes to improve some other traits of safflower; especially resistance to biotic and abiotic stresses. Cultivated species also had fewer days to flowering, which is a mechanism to avoid environmental stress. A high level of genetic diversity was also found among accessions of *C. tinctorius*, especially for seed yield and number of seeds per head as the most important components of seed yield. This diversity was predictable given the wide range of geographic origins and cultivation status of the germplasm that reflects the impact of climate, landscape, agricultural use, and history on the phenotypes of

the accessions. A similar agronomic evaluation on a small germplasm of cultivated safflower has also shown considerable diversity in similar traits (Amini et al., 2008). Majidi et al. (2011) reported that wild genotypes of *C. oxyacanthus* showed high degrees of drought tolerance and might be considered as useful sources for drought stress.

Molecular Analysis

Since 20 ISSR primers yielded 368 reproducible and polymorphic bands (72.4% polymorphism), this technique is a powerful tool for analysis of genetic diversity in safflower. The rate of ISSR polymorphism in safflower has been reported as 68.0 and 82.7% by Sehgal et al. (2009) and Yang et al. (2007), respectively. The mean value of PIC obtained in this study was 0.35, indicating that the ISSR marker is effective for characterization of genetic variation in the collection of safflower genotypes. The ISSR method can be seen as an intermediate between techniques such as RAPD-PCR and sophisticated techniques such as simple sequence repeat and amplified fragment length polymorphism. These markers represent a valuable resource for genetic analysis and basic evolutionary studies of safflower and will also be useful to help the taxonomic classification of genus *Carthamus* to deal with the problems of the current classifications (Vilatersana et al., 2005).

The results of cluster analysis, as well as PCA, showed a considerable agreement between taxonomic classification of *Carthamus* species and genetic variation by ISSR markers. These results indicated that ISSR markers could discriminate the seven examined species with the exception of *C. palaestinus*, which was not separated from *C. tinctorius* (Fig. 1 and 2). Previous studies have hypothesized that either *C. palaestinus* or *C. oxyacanthus* is the progenitor to the cultivated *C. tinctorius* (Ashri and Knowles, 1960; Ashri and Efron, 1964; Chapman and Burk, 2007). In the present study, the genetic similarities of *C. tinctorius* with *C. palaestinus* and *C. oxyacanthus* were 0.90 and 0.85, respectively (Table 5). Nevertheless, *C. oxyacanthus* accessions were clearly separated from cultivated safflower. These results support the hypothesis that *C. palaestinus* is most closely related to *C. tinctorius* and confirmed the microsatellite data by Bowles et al. (2010).

Distribution of wild species of *C. palaestinus* is limited to some areas of the Middle East including southern Palestine and northern Iraq–Iran. In this study, high genetic diversity among the safflower genotypes belonging to the Middle East center (Table 7) reinforces the probability of safflower originating from this region. Our findings are consistent with those of several studies that investigated the phylogenetic and genetic relationships between different species of safflower using data from morphological, cytogenetic, and molecular studies. Ashri and Knowles (1960) proposed that safflower was produced from the hybridization of *C. oxyacanthus* and *C. persicus*. According to Knowles and Ashri (1995), *C. oxyacanthus* and *C. palaestinus* can easily cross with *C. tinctorius*. Chapman and Burke (2007) assessed the DNA sequences of seven gene regions from 23 genotypes of safflower using single nucelotide polymorphism methods, and suggested that cultivated safflower had a closer relationship with *C. palaestinus* than with *C. oxyacanthus*. Likewise, Sasanuma et al. (2008) performed nucleotide sequence of nuclear SACPD gene and a Chloroplast region in 13 taxa of *Carthamus* and concluded that *C. arborescens* was the most primitive species in the genus and that *C. palaestinus* was genetically the closest to cultivated safflower.

Molecular Variation of *C. tinctorius* Accessions and Association of Gene Pools

Based on ISSR fingerprinting, the 79 cultivated safflowers were grouped into five subclusters (Fig. 1). Although this classification is in general agreement with available information regarding the origins of these genotypes, some accessions did not correlate with their geographic origin possibly due to heterogeneity, the dominant nature of ISSR marker, and migration (Roldán-Ruiz et al., 2000). Moreover, some accessions obtained from the regions other than centers of similarity and/or regional gene pools were clustered within the aforementioned groups. This may support available information on the history of human establishments and introduction of safflower from these gene pools to other regions in ancient times. For instance, inclusion of the accessions from Morocco and Libya in the European cluster (Cluster A_I; Table 1 and Fig. 1) supports the early introduction in these areas from France (Knowles, 1969). The clustering of Australian accessions with Egyptian and Sudanese accessions (Cluster A_{II}) may also confirm the fact that these genotypes were introduced from Egypt-Sudan region (Knowles 1969; Sehgal et al., 2009; Chapman and Burk 2007). Similarly, clustering of the accessions from the United States and Canada with those from the Middle East, especially Iran–Afghanistan gene pool, (Cluster A_{III}) may indicate the introduction of safflower to the Americas (Canada, United States, and Central and South America) from this region. It is noteworthy that samples from Iran–Afghanistan and Turkey were not separated from other areas of the Middle East. This supports the idea of Knowles (1969) and Sehgal et al. (2009) that Turkey should be treated as part of the Middle East gene pool. The samples belonging to the Far East (China and Japan), along with one accession from Uzbekistan, were isolated from the others as a separate cluster (Cluster A_V). Weiss (1971) believed that safflower was introduced into southern Russian Republics from the Iran–Afghanistan region and might have been then transferred to the Far East from Russian Republics such as Uzbekistan. According to Knowles (1969), safflower originated from the Middle East and was transferred to India–Pakistan (as a secondary center). It was later transferred to Europe and

elsewhere. In this study, the similarity between samples of the Middle East and India–Pakistan was high (0.91). The trends of safflower introduction from the most probable center (the Middle East and Iran-Afghanistan) to other gene pools can be illustrated using the dendrogram of 10 regional gene pools based on the ISSR data on 79 worldwide safflower accessions (Fig. 3).

The P, I, and He (Table 7) revealed the maximum and minimum genetic diversity in the Middle East and the Far East gene pool, respectively. This is in agreement with the findings of Sehgal et al. (2009). The high value of genetic diversity among genotypes of the Middle East may strongly confirm that safflower has originated from this region and has then been transferred to other regions. High similarity between Iran–Afghanistan and the Middle East centers (0.93, Table 6) may indicate that these two areas cannot be separated from each other in classification of safflower. Ashri and Knowles (1960) divided the Middle East center to three areas, including Iran–Afghanistan, Turkey, and Near East, according to morphological evaluations.

CONCLUSION

The results of the present study indicated that ISSR is a powerful tool and effective marker system for detecting genetic diversity among safflower genotypes. This marker system provided useful information about the relationships of the studied safflower species and their geographical regions. Our findings also confirmed that *C. palaestinus* is a closer relative of the cultivated safflower species than *C. oxyacanthus*. Both species are easily crossed with cultivated safflower and can be used as a new source of biotic and abiotic stress resistance genes in breeding programs. The level of genetic diversity among cultivated safflower detected in this study is sufficient enough for constructing mapping population, marker-assisted selection, and related genomic analysis of this important crop plant. With the structuring of genetic diversity using ISSR fingerprints, it was possible to clarify the genetic framework of "centers of similarity" or "regional gene pools." The ISSR markers and morphological descriptors can now be used as coherent tools for the development of core collections in safflower.

Acknowledgments

The authors would like to thank the USDA and the gene bank of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) for supplying seed samples. This work was supported by the Iran National Science Foundation (INSF) and Isfahan University of Technology, Iran.

References

Amini, F., G. Saeidi, and A. Arzani. 2008. Study of genetic diversity in safflower genotypes using agro-morphological traits and RAPD markers. Euphytica 163:21–30. doi:10.1007/s10681-007-9556-6

Archak, S., A.B. Gaikwad, D. Gautam, E.V.V.B. Rao, K.R.M. Swamy, and J.L. Karihaloo. 2003. Comparative assessment of DNA fingerprinting techniques (RAPD, ISSR and AFLP) for genetic analysis of cashew (*Anacardium occidentale* L.) accessions of India. Genome 46:362–369. doi:10.1139/g03-016

Ashri, A. 1975. Evaluation of the germplasm collection of safflower *Carthamus tinctorius* L. V. Distribution and regional divergence for morphological characters. Euphytica 24:651–659. doi:10.1007/BF00132903

Ashri, A., and Y. Efron. 1964. Inheritance study with fertile inter specific hybrids of three *Carthmus L.* species. Crop Sci. 4:510–514. doi:10.2135/cropsci1964.0011183X000400050023x

Ashri, A., and P.F. Knowles. 1960. Cytogenetics of safflower (*Carthamus L.*) species and their hybrids. Agron. J. 52:11–17. doi:10.2134/agronj1960.00021962005200010004x

Awasthi, A.K., G.M. Nagaraja, G.V. Naik, S. Kanginakudru, K. Thangavelu, and J. Nagaraju. 2004. Genetic diversity and relationships in mulberry (genus *Morus*) as revealed by RAPD and ISSR marker assays. BMC Genet. 5:1–9. doi:10.1186/1471-2156-5-1

Bowles, V.G., R. Mayerhofer, C. Davis, A.G. Good, and J.C. Hall. 2010. A Phylogenetic investigation of *Carthamus* combining sequence and microsatellite data. Plant Syst. Evol. 287:85–97. doi:10.1007/s00606-010-0292-3

Chapman, M.A., and J.M. Burke. 2007. DNA sequence diversity and the origin of cultivated safflower. BMC Plant Biol. 7:60–68. doi:10.1186/1471-2229-7-60

Chapman, M.A., J. Hvala, J. Strever, and J.M. Burke. 2010. Population genetic analysis of Safflower (*Carthamus tinctorius*; Asteraceae) reveals a Near Eastern origin and five centers of diversity. Am. J. Bot. 97:831–840. doi:10.3732/ajb.0900137

Chapman, M.A., J. Hvala, J. Strever, M. Matvienko, A. Kozik, R.W. Michelmore, S. Tang, S.J. Knapp, and J.M. Burke. 2009. Development, Polymorphism and cross-taxon utility of EST-SSR markers from safflower (*Carthamus tinctorius* L.). Theor. Appl. Genet. 120:85–91. doi:10.1007/s00122-009-1161-8

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin (version 3.0): An integrated software package for population genetics data analysis. Evol. Bioinform. 1:47–50.

Fufa, H., P.S. Baenziger, B.S. Beecher, I. Dweikat, R.A. Graybosch, and K.M. Eskridge. 2005. Comparison of phenotypic and molecular marker-based classifications of hard red winter wheat cultivars. Euphytica 145:133–146. doi:10.1007/s10681-005-0626-3

Galvan, M.Z., B. Bornet, P.A. Balatti, and M. Branchard. 2003. Inter-simple sequence repeat (ISSR) markers as a tool for the assessment of both genetic diversity and gene pool origin in common bean (*Phaseolus vulgaris L.*). Euphytica 132:297–301. doi:10.1023/A:1025032622411

Huang, C.Q., G.D. Liu, C.J. Bai, W.Q. Wang, S.Y. Zhou, and D.Q. Yu. 2010. Estimation of genetic variation in *Cynodon dactylon* accessions using the ISSR technique. Biochem. Syst. Ecol. 38:463–470. doi:10.1016/j.bse.2010.04.006

Knowles, P.F. 1969. Center of plant diversity and conservation of crop germplasm safflower. Econ. Bot. 23:324–329. doi:10.1007/BF02860678

Knowles, P.F., and A. Ashri. 1995. Safflower: *Carthamus tinctorius* (Compositae). In: J. Smartt and N.W. Simmonds, editors, Evolution of crop plants. Longman, UK. p. 47–50.

Kumar, H. 1991. Cytogenetics of safflower. Chromosome engineering in plants: Genetics, breeding, Evolution. Amesterdam, the Netherlands.

Mantel, N.A. 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27:209–220.

Mayerhofer, R., C. Archibald, V. Bowles, and A.G. Good. 2010. Development of molecular markers and linkage maps for the *Carthamus* species *C. tinctorius* and *C. oxyacanthus*. Genome 53:266–276. doi:10.1139/G10-002

Majidi, M.M., V. Tavakoli, A. Mirlohi, and M.R. Sabzalian. 2011. Wild safflower species (*Carthamus oxyacanthus* Bieb.): A possible source of drought tolerance for arid environments. Aust. J. Crop Sci. 5(8):1055–1063.

Mohammadi, S.A., and B.M. Prasana. 2003. Analysis of genetic diversity in crop plants salient statistical tools and considerations. Crop Sci. 43:1235–1248. doi:10.2135/cropsci2003.1235

Mundel, H.H., and J.W. Bergman. 2008. Safflower breeding. In: J. Vollmann and I. Rajcan, editors, Oil crop breeding. Springer series, Hand book of plant breeding, Springer, New York. p. 423–447.

Murray, M.G., and W.F. Thompson. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8:4321–4325.

Prevost, A., and M.J. Wilkinson. 1999. A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theor. Appl. Genet. 98:107–112. doi:10.1007/s001220051046

Reunova, G.D., I.L. Kats, T.I. Muzarok, and I. Zhuravleu. 2010. Polymerase of RAPD, ISSR and AFLP markers of the Panax ginseng CA Meyer (*Araliaceae*) genome. Genetika 46:1057–1066.

Rohlf, F.J. 1998. NTSYS-pc: Numerical taxonomy and multivariate analysis system. v.2.02 k. Applied Bio. Stat. NewYork.

Roldán-Ruiz, R., J. Dendauw, E.V. Bockstaele, A. Depicker, and M. Deloose. 2000. AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium spp.*). Mol. Breed. 6:125–134. doi:10.1023/A:1009680614564

SAS Institute. 1999. SAS/STAT users guide. SAS Institute, Inc, Cary, NC.

Sasanuma, T., D. Sehgal, T. Sasakuma, and S.N. Raina. 2008. phylogenetic analysis of Carthamus species based on the nucleotide sequence of nuclear SACPD gene and Chloroplast trn L-trn F IGS region. Genome 51:721–727. doi:10.1139/G08-059

Sehgal, D., V.R. Rajpal, and S.N. Raina. 2008. Chloroplast DNA diversity reveals the contribution of two wild species to the origin and evolution of diploid safflower (*Carthamus tinctorius* L.). Genome 51:638–643. doi:10.1139/G08-049

Sehgal, D., V.R. Rajpal, S.N. Raina, T. Sasanuma, and T. Sasakuma. 2009. Assaying Polymorphism at DNA level for genetic diversity diagnostics of the safflower (*Carthamus tinctorius L.*) world germplasm resources. Genetica 135:457–470.

Smolik, M., I. Ochmian, and J. Grajkowski. 2010. Genetic variability of Polish and Russian accessions of cultivated blue honeysuckle (*Lonicera caerulea*). Genetika 46:1079–1085.

Vilatersana, R., T. Garnatje, A. Sussana, and N. Garcia-Jacas. 2005. Taxonomic problem in *Carthamus* (Asteraceae): RAPD markers and sectional classification. Bot. J. Linn. Soc. 147:375–383. doi:10.1111/j.1095-8339.2005.00375.x

Weiss, E.A. 1971. Castor, sesame and safflower. Leonard Hill Books/University Press, Aberdeen, London.

Yang, Y.X., W. Wu, Y.L. Zheng, L. Chen, R.J. Liu, and C.Y. Huang. 2007. Genetic diversity and relationships among safflower (*Carthamus tinctorius* L.) analysis by inter-simple sequence repeats (ISSR). Genet. Resour. Crop Evol. 54:1043–1051. doi:10.1007/s10722-006-9192-3

Yeh, F.C., R. Yang, and T. Boyle. 1999. Popgene version 1.32: Microsoft Windows-based freeware for population genetic analysis. University of Alberta, Canada.

Zietkiewicz, E., A. Rafalski, and D. Labuda. 1994. Genome fingerprinting by simple sequence repeat (SSR) anchored polymerase chain reaction amplification. Genomics 20:176–183. doi:10.1006/geno.1994.1151