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RESEARCH ARTICLE

Genetic diversity and relationships among safflower (*Carthamus tinctorius* L.) analyzed by inter-simple sequence repeats (ISSRs)

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Abstract Genetic diversity and relationships among 48 safflower accessions were evaluated using 22 inter-simple sequence repeats (ISSR) primers. A total of 429 bands were amplified, and 355 bands (about 82.7%) were polymorphic. Five to forty-one polymorphic bands could be amplified by each primer, with an average of 16.1 polymorphic bands per primer. The results showed that the polymorphism of the safflower germplasm was higher at the DNA level. All the 48 accessions could be distinguished by ISSR markers and were divided into 9 groups based on ISSR GS by using UPGMA method. The genetic relationships among the accessions from different continents were closer. Comparatively, the genetic diversity of the accessions originated from Asia was higher, from Europe assembled. The results also showed that the genetic variation of accessions from Indian and Middle Eastern safflower diversity centers were relatively higher. ISSR is an effective and promising marker system for detecting genetic diversity among safflower and give some useful information on its phylogenic relationships.

Keywords *Carthamus tinctorius* L. · Genetic diversity · ISSR · Safflower

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Introduction

Safflower (*Carthamus tinctorius* L.) is one of the world's oldest oil seed crops. Currently, it has been grown commercially for edible oil and natural dye sources all over the world. Most of the genetic diversity that local and traditional varieties posses is being lost currently. The new variety are more genetically homogeneous and therefore more exposed to pathogens and adverse environmental conditions. Evaluation on the genetic diversity of safflower accessions originating from different gengraphical regions will help to provide valuable information on the conservation and ultization of safflower germplasm.

Inter-simple sequence repeats (ISSR) PCR using primers based on dinucleotide, tetranucleotide or pentanucleotide repeats has now become in fashion among the researchers (Zietkiwicz et al. 1994). For its advantages of simple procedure, low cost, good stability, high reproducibility and so on, ISSR marker has been successfully used in genetic mapping (Tanyolac 2003; Casaoli et al. 2001; Cekic et al. 2001),

germplasm identification (Nagaoka et al. 1997; Fang et al. 1998; Potter et al. 2002) and genetic diversity analysis (Wu et al. 2005; Joshi et al. 2000; Ash et al. 2003). Until now, few studies have been carried on the genetic variations of *C. tinctorius* L. using RAPD markers (Guo et al. 2003; Amiri et al. 2001) and isozymes (Zhang et al. 2000). The objective of the presented research here are (i) to assess the value of ISSR marker system for its ability to distinguish *C. tinctorius* L. accessions. (ii) to describe the genetic variation of *C. tinctorius* L. accessions from 32 different countries and to study their genetic relationships.

Materials and methods

Plant materials

Forty-eight safflower accessions from 32 different countries were analyzed (Table 1). Among them, 24 from Asia, 11 from Europe, 7 from Africa, 5 from America and 1 from Oceania.

DNA extraction

Genomic DNA was extracted from young leaves following the cetyltrimethylammonium (CTAB) procedure described by Saghai-Maroof et al. (1984) with minor modifications. The young leaves of 5–10 plants of each accession were ground to fine powder in liquid nitrogen. One to two grams of the powder was transferred to 15 ml prewarmed 2× CTAB DNA extraction buffer (100 mmol/l Tris–HCl pH 8.0, 10 mmol/l EDTA, 2% β-mercaptoethanol) in a 50 ml centrifuge tube, incubated in a 65°C water bath for 1–2 h, then taken out and cooled down to room temperature. The pretreated sample was mixed with equal volume of chlorform-isoamylalcohol (24:1) and put on ice for 30 min, then centrifuged for 15 min at 3500 rev/min. The aqueous phase was transfered to another tube and equal volume of absolute alcohol was added to precipitate the DNA. The DNA pellet was spooled out and washed with 70% alcohol twice or three times and 95% alcohol once, and then dissolved in TE (Tris–EDTA).

ISSR analysis

All the tested primers were synthesized by Shanghai Sangon Biotech Ltd., China according to Fang and Roose (1997) and Nagaoka and Ogihara (1997) (Table 2). Thirty primers were used to amplify the genome DNA of 2 accessions. Twenty-two of which could generate clear, repeatable and polymorphic bands were selected out for PCR amplification of the genomic DNA of 48 safflower accessions (Table 2).

The PCR volume was 25 µl, including 30 ng template DNA, 1 U Taq DNA polymerase, 1.5 mmol/l $MgCl_2$, 0.2 µmol/l primers, 200 µmol/l each of dNTP and 1× PCR buffer. PCR amplifications were performed in a PTC-220® thermocycler (MJ Research, USA). Initial denaturation was for 2 min at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at 50°C, 2 min at 72°C, with a final extension of 10 min at 72°C. The amplified products were separated on 2% agarose gels and stained with ethidium bromide. Images were photographed, captured by Gel Doc 2000TM (Bio-Rad, USA). When agarose electrophoresis resolved a complex band pattern, polyacrylamide electrophoresis was carried out. The amplified products were denatured at 95°C for 5 min, 10–12 µl were mixed with 5 µl of loading buffer with formamide. Then separated in 4% polyacrylamide gels (19:1 acrylamide *N*, *N*′methylenebisacrylamide) under denaturing conditions with 7.5 mol/l urea at 65°C, in a 1× TBE buffer, at maximum of 35 V/cm gel length and 75 W, for about 3 h, afterwards the gels was fixed in 10% (v/v) ethanol which contained 0.5% acetic acid solution and silver stained according to the procedure of Sanguinelti et al. (1994).

ISSR data scoring and analysis

The amplified DNA fragments were scored by presence (1) or absence (0) for each safflower accession. The data matrix was then used to calculate genetic similarity (GS) values according to Nei and Li's (1979) method:

$$GS = 2N_{ij}/(N_i + N_j)$$

Table 1 Accession numbers and its sources of the tested safflower

No.	Accession	Country	Continent	Diversity centers	No.	Accession	Country	Continent	Diversity centers
1	PI 253909	Afghanistan	Asia	Middle Eastern Center	25	PI 254976	Greece	Europe	European Center
2	PI 369842	Argentina	America	—	26	PI 253540	Hungary	Europe	European Center
3	PI 367833	Armenia	Asia	—	27	PI 312275	Hungary	Europe	European Center
4	PI 613517	Australia	Oceania	—	28	PI 306821	India	Asia	Indian Center
5	PI 253518	Austria	Europe	European Center	29	PI 306829	India	Asia	Indian Center
6	PI 301472	Bangladesh	Asia	Indian Center	30	PI 250009	Iran	Asia	Middle Eastern Center
7	PI 401474	Bangladesh	Asia	Indian Center	31	PI 613520	Iran	Asia	Middle Eastern Center
8	PI 401479	Bangladesh	Asia	Indian Center	32	PI 253758	Iraq	Asia	Middle Eastern Center
9	PI 253531	Bulgaria	Europe	European Center	33	PI 613454	Israel	Asia	Middle Eastern Center
10	PI 305539	Bulgaria	Europe	European Center	34	PI 209300	Kenya	Africa	Ethiopian Center
11	PI 559909	Canada	America	—	35	PI 576992	Korea, North	Asia	Far Eastern Center
12	PI 572544	Canada	America	—	36	PI 286199	Kuwait	Asia	Middle Eastern Center
13	Jianyanghonghua	China	Asia	Far Eastern Center	37	W6 18120	Mongolia	Asia	—
14	Chuanhong 1	China	Asia	Far Eastern Center	38	PI 195895	Morocco	Africa	European Center
15	Xinhong 1	China	Asia	Far Eastern Center	39	PI 248620	Pakistan	Asia	Indian Center
16	Xinhong 4	China	Asia	Far Eastern Center	40	PI 248628	Pakistan	Asia	Indian Center
17	Nanchuanhonghua	China	Asia	Far Eastern Center	41	PI 613534	Portugal	Europe	European Center
18	PI 250610	Egypt	Africa	Egyptian Center	42	PI 209287	Romania	Europe	European Center
19	PI 250081	Egypt	Africa	Egyptian Center	43	PI 239226	Spain	Europe	European Center
20	PI 286385	Eritrea	Africa	—	44	PI 181866	Syria	Asia	Middle Eastern Center
21	PI 286386	Eritrea	Africa	—	45	PI 613526	Turkey	Asia	Middle Eastern Center
22	PI 343930	Ethiopia	Africa	Ethiopian Center	46	PI 304499	Turkey	Asia	Middle Eastern Center
23	PI 253529	France	Europe	European Center	47	W6 908	United States	America	—
24	PI 253511	Germany	Europe	European Center	48	UC 26	United States	America	—

Classification of diversity centers according to citation in Yuan et al. (1989)

Table 2 ISSR primers used in this experiment and the results of the amplification of polymorphic primers

Primer	Sequence	Total amplified bands	Number of polymorphic bands	Percentage of polymorphic bands (%)	Primer	Sequence	Total amplified bands	Number of polymorphic bands	Percentage of polymorphic bands (%)
1	5′-HVH$(TG)_7$T-3′	—	—	—	16	5′-$(AG)_8$C-3′	31	25	80.6
2	5′-VHVG$(TG)_7$-3′	22	10	45.5	17	5′-$(GA)_8$T-3′	9	8	88.9
3	5′-BDB$(TCC)_5$-3′	46	41	89.1	18	5′-$(GA)_8$C-3′	36	29	80.6
4	5′-HVH$(TCC)_5$-3′	39	35	89.7	19	5′-$(CT)_8$G-5′	12	12	100
5	5′-DBDA$(CA)_7$-3′	—	—	—	20	5′-$(CA)_8$G-3′	10	10	100
6	5′-HVH$(CA)_7$T-3′	—	—	—	21	5′-$(GT)_8$A-3′	—	—	—
7	5′- BDB$(CA)_7$-3′	—	—	—	22	5′-$(TC)_8$C-3′	12	12	100
8	5′-DBD$(AC)_7$-3′	18	18	100	23	5′-$(TC)_8$G-3′	10	10	100
9	5′-$(TCC)_5$RY-3′	21	13	61.9	24	5′-$(AC)_8$C-3′	22	10	45.5
10	5′-$(CA)_8$RG-3′	—	—	—	25	5′-$(AC)_8$G-3′	20	20	100
11	5′-$(GA)_8$YG-3′	28	22	78.6	26	5′-$(TG)_8$G-3′	10	9	90
12	5′-$(AG)_8$YC-3′	15	10	66.7	27	5′-$(AG)_8$YT-3′	11	11	100
13	5′-$(AC)_8$YG-3′	13	12	92.3	28	5′-$(GA)_8$YC-3′	7	5	71.4
14	5′-$(GT)_8$YC-3′	—	—	—	29	5′-$(GA)_8$-3′	—	—	—
15	5′-$(AG)_8$T-3′	28	25	89.3	30	5′-$(CT)_8$RG-3′	9	8	88.9
Total		429	355		Average		19.5	16.1	82.8

R = A/T, Y = G/C, B = T/G/C; D = A/T/G, H = A/T/C, V = 3A/G/C.

The primer 1–14 synthesized according to Fang and Roose (1997)

The primer 15–30 synthesized according to Nagaoka and Ogihara (1997)

where N_{ij} is the number of ISSR bands in common between accessions i and j, and N_i and N_j are the total number of ISSR bands observed for accessions i and j, respectively. Based on the GS matrix, a dendrogram showing the genetic relationships between accessions was constructed using the unweighted pair-group method with arithmetic average (UPGMA) through the computer software NTSYS-pc version 2.10 (Rohlf 1993).

Results

ISSR polymorphisms

A total of 429 bands were scored for 22 ISSR primers (Table 2), with an average of 19.5 bands per primer, 355 out of 429 bands (82.7%) were polymorphic, with an average of 16.1 polymorphic bands per primer. A range of 7–46 bands or 5–41 polymorphic bands were detected by each primer. The size of the band ranged from 200 bp to 2000 bp. Fig. 1 illustrated the example of amplified products with ISSR primer 19. These results indicate that ISSR markers detected a higher level of polymorphism among the 48 safflower accessions.

Genetic similarity

About 355 polymorphic bands were analyzed for GS among the 48 safflower accessions. It was showed that the GS values ranged from 0.724 to 0.970, with an average of 0.827. The GS value between PI 286386 and PI 343930 was the highest and that between PI 253909 and W6 18120 was the lowest.

The average GS values and their variation coefficients within and between the accessions from the different continents were presented in Table 3 (the data of Oceania was not presented for its only one accession). The average GS value within the accessions of the different continents was 0.839, while that between them was 0.833. Actually, the average GS value within the accessions from Asia, Europe, Africa and America were quite similar (0.820, 0.852, 0.856, 0.828, respectively), and its coefficients of variation (CV) (4.62%, 3.60%, 4.96% and 4.30%, respectively) were also similar, which inferred that the genetic relationships among the accessions from different continents were closer.

Further analysis showed that the average GS value within the accessions from Asia (0.820) and that between the accessions from Asia and other

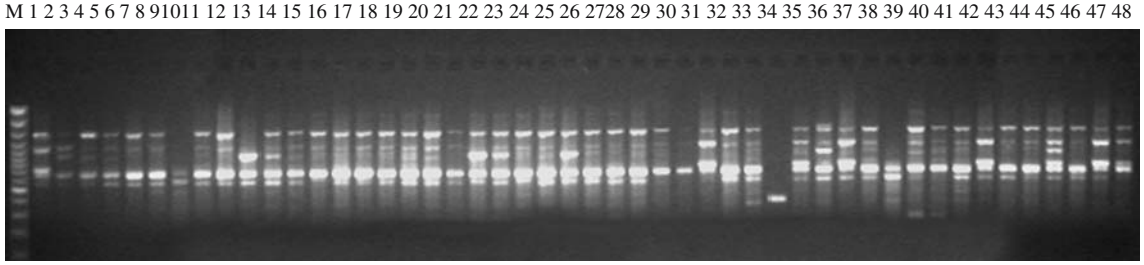

Fig. 1 The amplification pattern of primer 19

Table 3 Average value of genetic similarity (GS) and its variation coefficient (CV) within and between the accessions from different continents based on ISSR markers

Genetic similarity (GS)	Asia	Europe	Africa	America
Asia	0.820 (4.60%)			
Europe	0.826 (4.28%)	0.852 (3.61%)		
Africa	0.824 (4.45%)	0.846 (3.56%)	0.856 (4.96%)	
America	0.821 (4.36%)	0.842 (3.50%)	0.837 (4.10%)	0.828 (4.30%)

Numbers lined were average values of genetic similarity within continents, the others were average values between two continents. Numbers in the brackets were variation coefficient of GS accordingly

continents (0.826, 0.824 and 0.821) were all lower than that among all the accessions (0.827). And the average GS values within the Asian accessions were lower than that between the accessions of Asian to other continents. These results indicated that the genetic diversity of the Asian accessions was relatively plentiful. The average GS values within the European and African accessions were higher than that between them to other continents' accessions. Especially for the European accessions, the CV of GS within them, between them to African accessions, and between them to American accessions were all relatively lower and closer, with the value of 3.61%, 3.56% and 3.50%, respectively, while the CV of GS between European to Asian accessions was 4.28%. It was inferred that the genetic relationship within the European accessions was relatively closer and it was more different from Asian accessions. The average GS value between American to European accessions was the highest and its variation coefficient was the lowest which suggested American accessions be quite similar to European accessions.

Clustering analysis

The genetic relationships among 48 safflower accessions based on ISSR markers were estimated by UPGMA cluster analysis of the GS matrix (Fig. 2). All the 48 accessions could be distinguished by 22 ISSR markers. Using the average GS value among all safflower accessions as threshold, 48 safflower accessions could be divided into 9 groups. The group I included PI 253909 and PI 369842. The group II was composed of nine accessions, including PI 367833, PI 253518, PI 401479, and PI 305539 and so on. Among them, two accessions came from Asia, three from Europe, three from America, another one from Oceania. The group III was composed of 29 accessions (i.e. PI 195895, PI 209287, PI 248620, and UC 26). Of them, 13 accessions came from Asia, 8 from Europe, 2 from America, and 6 from Africa. The group IV was composed of three accessions, all came from southwest of China, including Jianyanghonghua, Chuanhong 1 and Nanchuanhonghua. PI 613454 (Israel), W6 18120 (Mognolia), PI 306829 (India), PI 401474 (Bangladesh) all from Asia and PI 250081 from Egypt was separated into solely group by itself (groups V–VIIII). From the dendrogram, it was clearly showed that some accessions originated from the same country or district was closely assembled together, such as W6 908 and UC 26 from the USA, PI 559909 and PI 572544 from Canada, PI 253540 and PI 312275 rooted in Hungary, PI 248620 and PI 248628 from Pakistan and so on all could be closely clustered into one group, respectively.

Knowles put forward that there were seven safflower diversity centers all around the world in 1969, including Far Eastern, Indian, Middle Eastern, Egyptian, Sudan, Ethiopian and European Center (literature cited in Yuan et al. 1989). The results in this experiment showed that six accessions from Far Eastern Center including five from China were clustered into groups III and IV. Seven accessions in Indian Center were relatively scattered into groups II, III, VII and IV. Among them, two accessions rooted in Pakistan were closely clustered into one group, while three accessions from Bangladesh and two from India were scattered into different groups. Nine accessions belonged to Middle Eastern Center were seperated into groups I, III and V. Of them, seven accessions were classified into group III, the others were into other two groups. Two accessions of Egyptian Center were dispersed into groups III and VI. But ten accessions of European Center were relatively clustered into groups II and III, seven accessions of them classified into one group, the other three accessions clustered into another group. Five accessions from America were comparatively concentrated into groups II and III.

Discussion

Effectiveness of ISSR markers in evaluating safflower's diversity

ISSR could reveal a higher level of polymorphism in previous studies. This markers have been used successfully in estimating the genetic diversity in rice (Blair et al. 1999), gooseberry (Eiadthong et al. 1999), *Hottuynia* Thunb. (Wu et al. 2005),

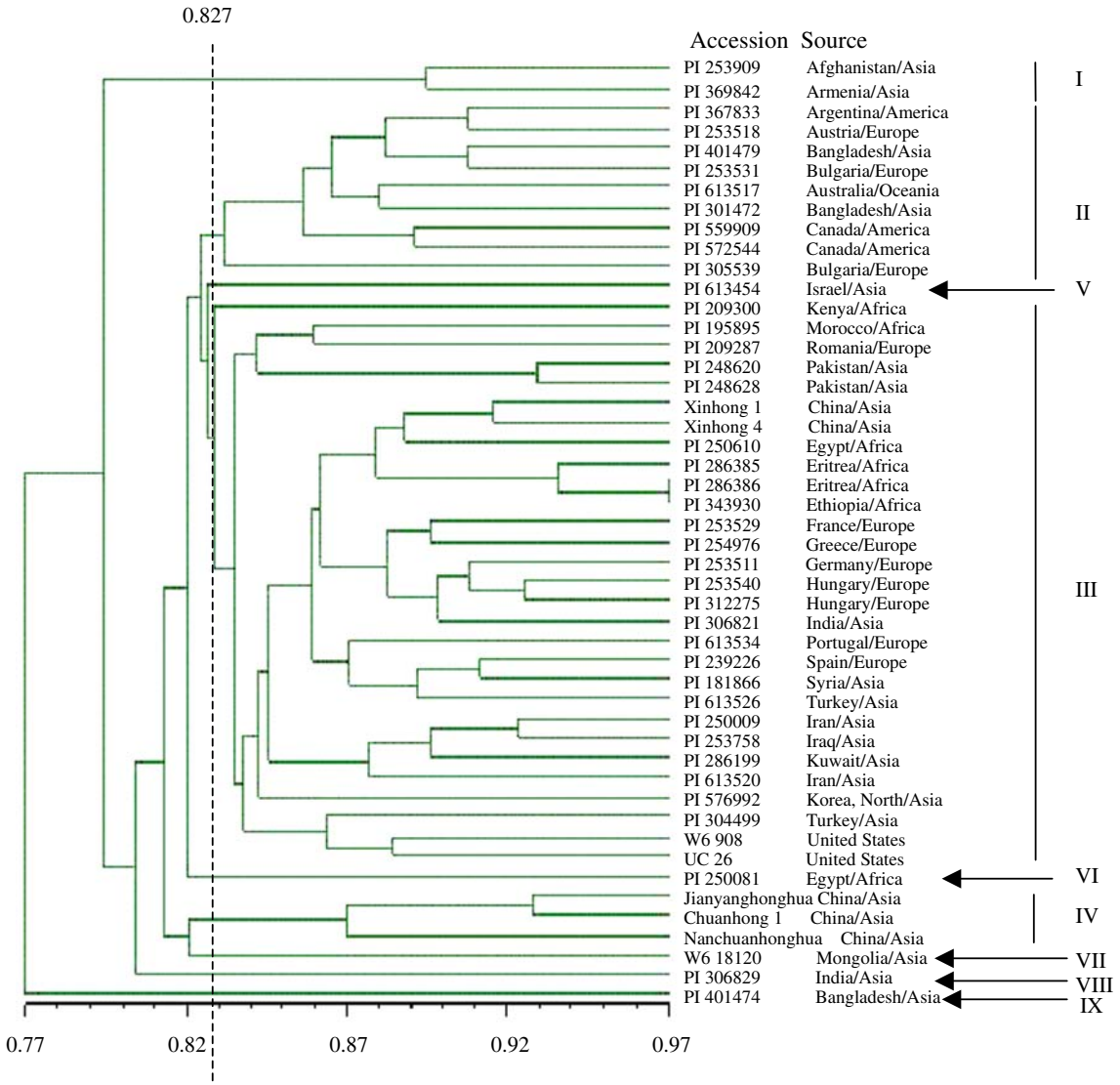

Fig. 2 UPGMA dendrogram of 48 safflower accessions based on ISSR genetic similarities

Oryza granulate (Qian et al. 2000), pawpaw (Pomper et al. 2003), buffalograsses (Budak et al. 2004). The studies of Qian et al. (2000), Budak et al. (2004) and Wu et al. (2005) indicated that ISSR could detect more genetic variation than that of RAPD. In this study, a total of 429 bands were amplified from 22 primers while 355 bands (about 82.7%) were polymorphic and 73.3% primer could produce polymorphism. The results indicated that ISSR markers could reveal a higher level of polymorphism among 48 safflower accessions. The cluster results also showed that all the accessions could be distinguished by ISSR markers, which laid a solid foundation for evaluating the genetic diversity of safflower.

The relationship between clustering results and geographical distribution

In this study, 24 accessions from Asia were scattered into each group, especially the accessions from India, China and Bangladesh, which indicated that the genetic diversity of Asian accessions were higher. Comparatively, the accessions originated from Europe were relatively assembled.

These results are in agreement with the result of Zhang (2000) obtained by using isozyme technique. Knowles inferred that safflower diversity centers were the similar centers rather than the original centers. Vavilov considered that there were three safflower centers, including Indian Center, based on its plentiful germplasm resources of safflower and longtime cultivation; the second was Afghanistan Center due to the variation of safflower was similar to the wild species of safflower, the third was Egyptian Center, because it was regarded as the original regions appearing the wild safflower (literature cited in Yuan et al. 1989). Our study indicated that the genetic variations of the accessions came from Indian and Middle Eastern were higher (We can not discuss the situation of Egyptian Center as only two accession from it were analyzed in this paper), while the accessions from the other centers were genetically assembled comparatively. These results inferred that Indian and Middle Eastern Centers might be the original centers of safflower, while the others be the similar centers. To convince it, some more accessions need to be analyzed and some more experiments need to be conducted.

China has plentiful safflower resources with its 2100 years of safflower cultivation history and long-term selection and breeding. Five accessions came from China were classified into two groups. Xinghong 1 and Xinghong 4 originated from Xinjiang Minority National Municipality of China were clustered into one group, while Jianyanghonghua, Chuanhong 1 and Nanchuanhonghua from southwest of China were classified into another group. Actually, both Xinghong 1 and Xinghong 4 have AC-1 in their parentage. And Chuanhong 1 was the variety selected from Jianyanghonghua, a landraces of Jianyang areas of Sichuan province. Nanchuanhonghua was also collected at Jianyang areas. They might have close consanguinity. Guo et al. (1999, 2003) found that there were significant differences between different safflower accessions in China at their morphological characteristics, compositions and contents of chemical contituents, even at the DNA level. Although only five accessions used in this study, the higher genetic diversity still could be detected from the accession came from different regions of China in spite of the lower genetic difference between the accessions from the same regions. It was inferred that although China not the original center of safflower, it has plentiful safflower resources at the different areas of China with its long cultivation history, and it now becomes one of the cultivation centers of safflower in the world.

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