PROGRESS IN BIOLOGICAL SCIENCES

Relationship among AFLP, RAPD marker diversity and Agromorphological traits in safflower (*Carthamus tinctorius* L.)

Bahman Panahi[1,*], Rahim Afzal[2], Mahmoud Ghorbanzadeh Neghab[2], Mohsen Mahmoodnia[3], Behzad Paymard[1]

[1]Department of Biotechnology and Plant Breeding, University of Tabriz, Iran.
[2]Department of Biotechnology and Plant Breeding, Ferdowsi University of mashhad, Iran
[3]Department of Agronomy and Plant Breeding, Valieasr University of Rafsanjan, Iran

Received: February 10, 2013; Accepted: March 21, 2013

Abstract

Safflower *(Carthamus tinctorius*), is widely cultivated in agricultural systems as a source of high-quality vegetable and industrial oil. 9 RAPD primers, 6 AFLP primer combinations and 12 agro-morphological traits were used to assess the genetic diversity of 20 accessions of safflower representing global germplasm variability. Jacquards' similarity coefficient were used to understanding the genetic relationships among accessions, for AFLP and RAPD markers and Euclidian similarity coefficient for agro-morphological markers. UPGMA clustering algorithm was used for all markers. RAPD and AFLP markers grouped accessions into two main clusters whereas dendrogram of morphology data delineated the accessions into three clusters. Correlation coefficient comparisons between similarity matrices and co-phenetic matrices obtained with the three markers revealed that AFLP displayed no congruence vis-a-vis RAPD and agro-morphological data.

Keywords: AFLP, *Carthamus tinctorius* L., Genetic diversity, RAPD, marker

Introduction

Despite the large volume of globally traded vegetable oils, 75% of the production is from only four crops: soybean, oil palm, rapeseed and sunflower (1). Because of the domination of these four (2) many other oil crops are now underutilized or neglected, though these species provide opportunities through their great genetic diversity and diverse agro-ecological adaptation (3). *Carthamus tinctorius* L. commonly known as safflower is a member of the Asteraceae familyis a multi-purpose plant which is cultivated in more than 20 countries. Traditionally, this crop was grown for its yellow-orange flowers which were used for coloring and flavoring foods, and making dyes (4, 5) Safflower oil is thought to be one of the highest quality vegetable oils, containing oleic acid and linoleic acid (1). For the effective use of underutilized crops, it is critical to understand the extent and distribution of genetic diversity within species (6). Unfortunately, most of the genetic diversity of this plant currently is being lost so the evaluation of genetic diversity will help to provide valuable information on the management and utilization of safflower germplasm (7).

So far germplasm resources of safflower have been characterized on the basis of agro-morphological traits (8-10), biochemical characters (11) and molecular markers such as AFLP, SSR and ISSR (12-14). Mohammadi and Parsana (15) suggested that genetic diversity of safflower is the best estimated if agro-morphological and molecular marker studies are used together. Nevertheless, less report are presented for safflower using DNA based markers such as random amplified polymorphic DNA (RAPD), sequence-related amplified polymorphism (SRAP) and amplified fragment lengths polymorphism (AFLP) and its integration with agro-morphological traits (1).

*Corresponding author: Panahibahman@ymail.cm ir
Tel.: +98-0914-7029760

Therefore, the objectives of this research are evaluation of genetic diversity using AFLP, RAPD and their relationships with agro-morphological traits in international safflower germplasm.

Materials and methods

Plant material

The collection of promising genotypes for different purposes was obtained from several seed banks and sown in April 2011 in the experimental field at Faculty of Agriculture in University of Tabriz, Iran. The experiment was done in a Randomized Complete Block Design. In every block, there were three rows and in each rows, 25 seeds were sown. Each row was 3.5 m long, and the distance between rows was 50 cm. 20 accessions were used in this study. Names and origins of accessions are shown in Table 1.

Table 1. Accessions of *Carthamus tinctorius* L. used in this study, their origin and names.

No.	Name	Accession	Origin
1	MEX1	Quiriego-88	Mexico
2	MEX2	Sahuaripa-88	Mexico
3	MEX3	Bacum92	Mexico
4	CAN1	Saffire	Canada
5	CAN2	Lesaf	Canada
6	EGY1	PI-250536	Egypt
7	EGY2	PI-250537	Egypt
8	USA1	Hartman	USA
9	USA2	Finch	USA
10	CIM1	S-0023	Cimmyt
11	CIM2	VF-18	Cimmyt
12	IRN1	Local Ghochan1	Iran
13	IRN2	Local Ghochan2	Iran
14	IRN3	Local Isfahan1	Iran
15	IRN4	Local Isfahan2	Iran
16	IRN5	Local Marand	Iran
17	IRN6	Local Darab	Iran
18	IRN7	IL-111	Iran
19	SYR	Syrian	Syria
20	Wild	*C. oxycantha* (Mashhad)	Iran

Agro-morphological data collection and analysis

For multivariate and other analysis, twenteen variables were measured as described below:

1- Days to flowering (DF) was the date when the first flower bloomed,
2- Blooming time (BT) was the date when 50% of flowers were opened.
3- Plant height (PH) (cm)
4- The firs branch height (BH) (cm)
5- The number of secondary branch (NSB)
6- Tributaries angle (TA) (degree)
7- Number of heads (NH)
8- Number of seed per head (NSH)
9- Thousand seed weight (TSW)
10- Oil percentage (OP), measure with Soxhlet extractor (velp, Italy)
11- Shell percentage (SP)
12- Seed yield (SY) (kg/hec)

Analysis of morphological data

Pearson correlation coefficient was determined by JMP4 software. The means of each agro-morphological trait were used for cluster analysis. Agro-morphological data was standardized before using in multivariate analysis by applying the YBAR and STD options in NTSYS-pc software version 2.1 (16). Euclidean distance was used as the similarity coefficient for cluster analysis with the Unweighted Pair Group Arithmetic Means method (UPGMA) using NTSYS-pc software version 2.1 (16). Principal component analysis (PCA) was then performed on all these variables to certain that the dendrogram is a good representation of the data. Correlations between the distance and similarity matrices were performed using MXCOMP option in NTSYS-pc software version 2.1 (16).

DNA extraction and RAPD fingerprinting

For DNA extraction, young leaves were pooled together from ten individual plants of each cultivar. Total genomic DNA was extracted by Bioneer DNA Extraction Kit according to manufacture protocol. DNA concentration was quantified by using the NanoDrop Spectrophotometer 2000 (Thermoscientific, Germany) and qualified using agarose gel electrophoresis (Bio-Rad, USA).

Thirty random decamer primers tested and nine pair primers were selected for RAPD fingerprinting (Table 2). The reaction mixture of 25 µl volume contained 10X 2.5 µl assay buffer, 0.24 mM dNTPs, 15 ng primer, 0.5 U Taq DNA polymerase, 20 ng DNA template, and 1.5 mM $MgCl_2$ (Fermentase). DNA amplification was performed in a Bio-Rad thermal cycler programmed to 1 cycle of 4 min at 94°C followed by 40 cycles 94°C for 1 min, 35°C for 1 min, 72°C for 2 min ending with 1 cycle of 10 min at 72°C (final extension).

AFLP fingerprinting

Amplified fragment length polymorphism (AFLP) genotyping was performed using Vos et al. (17) methods. Digestion and ligation of genomic DNA carried out as Sehgal and Raina (12) described using EcoRI and TaqI enzymes (Fermentase, USA). The pre-selective amplification was done with *Eco*RI and *Taq*I primers which hadn't selective nucleotides. Thermo-cycler program for pre-selective reactions was 28 cycles of 94°C for 30 s, 52°C for 45 s, 72°C for 60 s, followed by 72°C for 60 s. Amplification confirmed using agarose gel electrophoresis. Selective amplification was done with 6 primers combination (Table 2) which thermo-cycler program was 13 cycles of 94°C for 30 s, 63°C for 30 s with a temperature decrease of −0.7°C per cycle, 72°C for 60 s; followed by 22 cycles at 94°C for 30 s, 54°C for 30 s, 72°C for 60 s, and finally 72°C for 7 min.

Separation and visualization of the AFLP markers was done on 6%poly acryl amide and for silver staining, gel was fixed in 10% (v/v) ethanol and 0.5% (v/v) acetic acid for 30 min then was rinsed three times in de ionized water. Then the gel was stained plate for 30 min in a solution containing 2 g L^{-1} of silver nitrate. The stained plate was rinsed with de ionized water for 5 s and developed in a pre chilled (4°C) developer solution containing 15 gL^{-1} of sodium hydroxide, 4.8 mlL^{-1} of 37% formaldehyde. When the bands became visible, the gel was immediately transferred to fixative solution to stop further reaction. The gel was finally rinsed with distilled water and air dried.

Table 2. RAPD and AFLP primers, total produced bands, polymorphic bands, with sequence used to assess genetic diversity of safflower.

Primer	Sequence 5'-3'	Total produced bands	Polymorphic bands	Polymorphic%
		RAPD		
UB30	CCGGCCTTAG	14	11	78.5
UB12	CCTGGGTCCA	12	10	91.6
UB89	GGGGGCTTGG	10	3	40
UB79	GAGCTCGTGT	12	8	66.6
UB96	GGCGGCATGG	18	11	61.1
UB25	ACAGGGCTC	9	3	33.3
UB91	GGGTGGTTGC	8	7	87.5
UB18	GGGCCGTTT	12	9	75
UB67	GAGCACCAGT	16	9	56.2
Mean		12.3	8.11	65.53
		AFLP		
(*Eco*+GA)+(*Taq* +AC)		42	34	80.95
(*Eco* +GA)+(*Taq*+TA)		36	21	58.33
(*Eco*+GA) + (*Taq*+TC)		35	19	54.28
(*Eco*+AG)+(*Taq* +CA)		28	13	46.42
(*Eco*+TG) + (*Taq* +TG)		45	31	68.88
(*Eco*+TG)+ (*Taq*+TT)		52	36	69.23
Mean		39.66	25.66	63.01

Molecular data analysis

Amplified products were scored for the presence (1) or absence (0) of bands and binary matrices were assembled for the two AFLP and RAPD markers. The binary matrices were subjected to statistical analyses using NTSYS-pc software version 2.1 (16). Jacquard similarity coefficient was employed to compute pair wise genetic similarities. For each marker the similarity matrix was used for the cluster analysis and construction of dendrogram using Un weighted Pair-Group Method (UPGMA) (18) and for principal coordinate analysis (PCA) (14) using the NTSYS-pc version 2.1 (16). To check the goodness fit of a cluster analysis to the associated similarity matrix, Co-phenetic Correlation Coefficient (CCC) was calculated for all the markers employed. Degree of congruence between RAPD and AFLP marker was ascertained by Mantel matrix correspondence test (19), a randomization procedure that compares the correlation between two matrices.

For individual primer/primer combination and regional group, number of polymorphic bands (P), polymorphism percentage (%P), and number of

banding patterns (N) were calculated using Pop gene software. Effective number of patterns per assay unit (P) determines the ability of a marker system on per assay basis to distinguish number of individuals in a population (20), was calculated as Ae = 1⁄(1 - h) = 1⁄Σpi 2 Where, pi is frequency of the i allele in a locus and h = 1-Σpi^2 th is heterozygosity in a locus. This is an extension of polymorphic information content (PIC) (21) available from frequencies of different banding patterns generated by a primer/primer combination. The Shannon's diversity index for each regional accession was calculated as H pop = -∑pilog p, where p is the frequency of a given band in a regional accession.

Also two marker systems as a whole were characterized by Nei's genetic diversity (D), probability that at a single locus chosen at random from the regional accessions are different to each other, calculated as hj = 1-p^2-q^2 Where, hj is average expected heterozygosis , q and p are allele frequencies. The non-parametric analysis of molecular variance (AMOVA) described by Excoffier et al. (22) was used to partition the variation within and between regional accessions by AFLP and RAPD marker systems.

Results

Correlations among variables

Mean, minimum, maximum and standard error of measured variables shown in Table 3. As shown in Table 4, there was a significant (P<0.01) correlation between days to flowering and plant height as well as plant height and blooming time. The number of second brancheshad positively correlated with blooming time, and negatively with both days to flowering and plant height (P<0.01) (Table 4).

A high significant negative correlation existed between the thousand seed weight and first branch high. This relationship has also been reported by other authors (23).

The results showed that plant yield has a positive and significant correlation with number of heads per plant (0.35) and number of secondary branches (0.33) (Table 4).

Table 3. Agro-morphological variations in 20 safflower germplasm accessions, minimum, mean, maximum and standard error. Variable abbreviations are according to Table 1.

No.	Variable	Min	Mean	Max	Std E
1	DF	82.8	84.7	87.7	1.47
2	BT	86.2	88.6	91.7	1.61
3	PH	83.4	92.6	102.8	5.47
4	BH	46.1	57.9	73.9	6.59
5	NSB	4.8	5.8	6.9	0.58
6	TA	34.8	38.7	48.8	3.1
7	NH	7	9.7	11.2	0.97
8	NSH	24.7	32	42.9	5.3
9	TSW	3.5	4.1	5	0.38
10	OP	23.5	29.9	37.4	3.1
11	SP	31.3	41.8	55	6.1
12	SY	1491	2044	2656	297

Table 4. Pearson correlation coefficients for agro-morphological traits of 20 safflower germplasm. *: Significant at 5% level. **: Significant at 1% level.

	DF	BT	PH	BH	NSB	TA	NH	NSH	TSW	OP	SP	SY
DF	-											
BT	0.98**	-										
PH	0.71**	0.73**	-									
BH	0.74**	0.76**	0.71**	-								
NSB	0.13	0.14	0.07	-0.10	-							
TA	0.17	0.16	0.15	0.30	-0.15	-						
NH	0.42	0.47*	0.27*	0.13	0.45*	0.15	-					
NSH	0.51*	0.53*	0.45	0.69**	0.12	0.26	0.16	-				
TSW	-0.73**	-0.74**	-0.70**	-0.91**	0.04	-0.32	-0.16	-0.70**	-			
OP	0.10	0.09	0.08	-0.02	0.47*	-0.37	-0.03	0.23	-0.09	-		
SP	0.36	0.43	0.42	0.12	-0.12	0.16	0.19	-0.15	-0.17	0.06	-	
SY	0.06	0.07	0.11	0.36	0.27	-0.17	0.35*	0.33*	-0.23	-0.02	-0.20	-

RAPD fingerprinting

Figure 1a shows RAPD profiles of 20 accessions obtained with RAPD primers UB67. Nine RAPD primers produced a total of 78 bands across the 20 accessions of *Carthamus tinct orius* ranging from 18 (UB 96) to 8 products (UB 91). The molecular size of the bands ranged from 250 to 3,500 bp. The average frequency of bands per primer was 20. Also, the number of polymorphic products (N) ranged from 3 to 11 with an average frequency of 7.8 per primer (Table 1).

The six primer combinations produced 238 bands with 39.6 bands per combination. The number of score able bands per primer combination ranged from 52 (Eco+AG/Taq+CA) to 52 (Eco+TG/ Taq+TT) with an average of 39 (Table 1). The polymorphic bands of six combinations ranged between 13 and 36 with an average of 25 bands per combination (Table 1). Figure 1b shown the profile of 20 accessions obtained with (*Eco*+GA) / (*Taq*+TC) primer combinations.

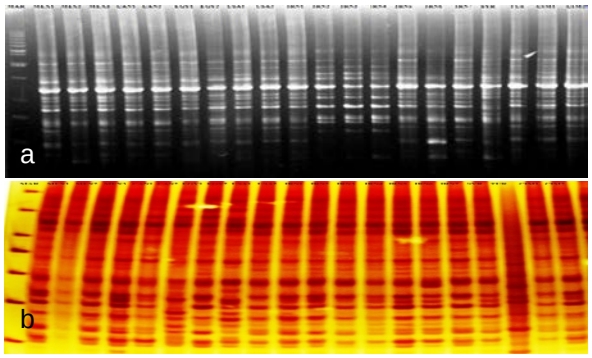

Figure 1. analysis of 20 safflower accessions from different geographical regions, RAPD **(a)** with primer UB67 and AFLP **(b)** produced with primer combination (*Eco*+GA)/(*Taq*+TC) (see Table 2). Marker is 100 bp.AFLP fingerprinting

Cluster analysis

Pair wise similarities regard to AFLP, RAPD and morphological markers ranged from 0.57 to 0.93, 0.30 to 0.79, and 19.5 to 1165.05, respectively. The clustering pattern obtained with RAPD, AFLP and morphological data showed distinctive pattern of 20 accessions (Fig. 2 a-c). The twodendrogram of RAPD and AFLP markers grouped 20 accessions into two main clusters whereas dendrogram of morphology data delineated the 20 accessions into three clusters of 6, 12, and 2 accessions without ungrouped accession. First cluster in RAPD based dendrogram had accessions MEX1, MEX3, MEX2, CIM2, USA2, CIM1, CAN2, IRN7, SYR, EGY2, IRN4 and CAN1 while in AFLP based dendrogram first cluster was constituted by MEX1, CAN1, MEX3, CAN2 and USA2. Wild genotype was genetically the most distinct cultivar in both AFLP and RAPD based dendrogram. Three genotypes of CAN1, MEX3 and CAN2 were grouped together in the entire three dendrogram. Regard to the remaining seventeen accessions, the phonograms based on AFLP data was quite distinct compared to the one based on either RAPD or morphological data (Fig. 2a-c).

The cophenetic correlation co-efficient (CCC) for RAPD, AFLP, and morphology data was r = 0.83, r = 0.91, and r = 0.93, respectively, suggesting a good fit between the dendrogram and the corresponding similarity. The mantel matrix test, however, showed no correlation (r>0.5, P = 0.01) among the similarity matrices obtained with the three marker types.

Partitioning of genetic and morphology variation

Relationship among 20 *Carthamus tinctrius* accessions were also visualized by performing principle coordinate analysis (PCA) based on morphological, AFLP and RAPD data. Correlation between the original variables and the first 3 principal components is shown in Table 5. The first results from the PCA of morphological data indicated that more than 77% of the variability observed can be explained by the first three components (Table 5). Correlation between principal components (PC1) contributed about 39% of the variation, showing highest contributions from the proportions of days to flowering and blooming time, PC2 contributed 14% of the total variation, consisted number of secondary branch and number of head contributing the most (Table 6). On PC3, the largest scores were due to yield and shell percentage. In two molecular markers, principle correlation showed that the first six Eigen values accounted 79.33 and 92.94% of variation in RAPD and AFLP, respectively (Table 7). Two-dimensional plot generated from PCA for

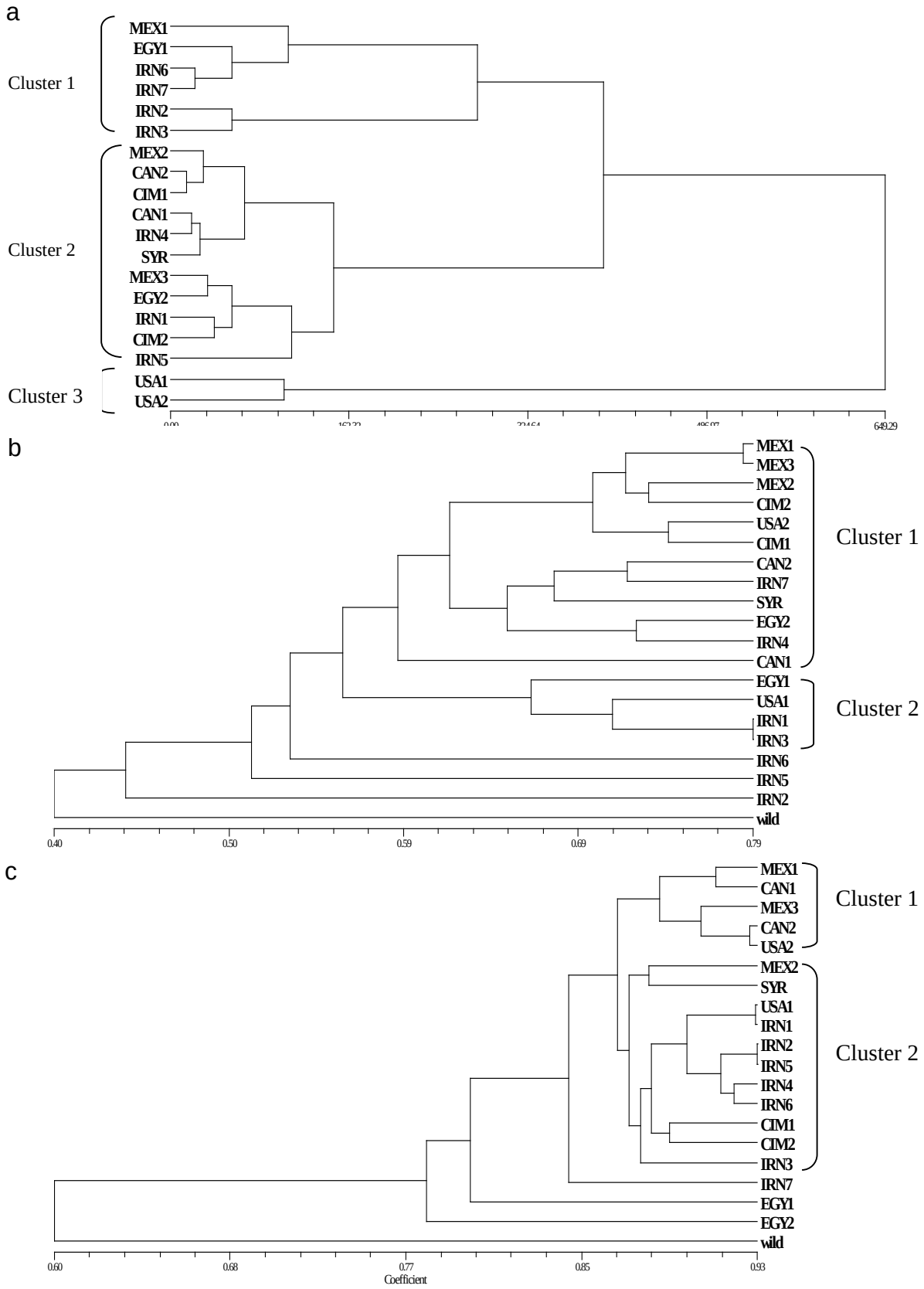

Figure 2. Dendrogram for 20 safflower accessions generated by UPGMA clustering using Jacquard's coefficient of similarity on agro-morphology **(a)** RAPD **(b)** and AFLP **(c)** data.

Table 5. Eigenvalues and proportion of total variability among sofflower genotypes as explained by the first four principal components.

Axis	Eigenvalue	Percent variance	Cumulative
1	4.73	39.45	39.45
2	1.78	14.8	54.25
3	1.46	12.2	66.45
4	1.36	11.35	77.79

Table 6. Correlation between the original variables and the first three principal components (PC).

No.	Variable	PC1	PC2	PC3
1	DF	0.40	0.07	-0.20
2	BT	0.41	0.12	-0.21
3	PH	0.39	-0.01	0.05
4	BH	0.38	-0.27	0.06
5	NSB	-0.01	0.66	0.27
6	TA	0.12	0.03	-0.17
7	NH	0.17	0.45	0.10
8	NSH	0.34	-0.14	0.37
9	TSW	-0.40	0.04	-0.09
10	OP	0.05	0.42	0.22
11	SP	0.19	0.15	-0.55
12	SY	0.11	-0.22	0.54

Table 7. Eigenvalues, percentage and cumulative proportions for 6 principal coordinate axes, derived from RAPD and AFLP markers application on 20 accessions of *Carthamus tinctorius* L.

Axis	Eigenvalue	Percent	Cumulative
RAPD			
1	11.7	58.56	58.56
2	1.19	5.94	64.5
3	0.87	4.37	68.88
4	0.8	4.04	72.92
5	0.68	3.41	76.34
6	0.59	2.98	79.33
AFLP			
1	16.87	84.35	84.35
2	0.58	2.9	87.25
3	0.37	1.84	89.1
4	0.29	1.47	90.57
5	0.28	1.41	91.9
6	0.19	0.95	92.94

RAPD and AFLP also supported the clustering pattern of UPGMA dendrogram (Fig. 3a-b).

The variation between and within the regional accessions was calculated by Shannon's index (based on AFLP and RAPD data) (Table 8). In addition, AMOVA indicated that big portion of genetic variation is corresponded to differences among individuals (Table 9). Proportion of diversity within and between regional accessions based on AFLP and RAPD data was 100, 0 and 90, 10%, respectively (Table 9).

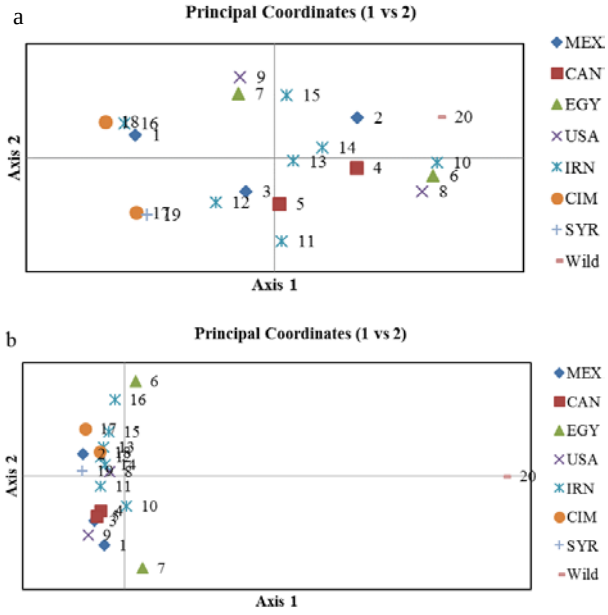

Figure 3. Principle coordin ate analysis for RAPD primers **(a)** and AFLP primers **(b)** applied on 20 *Carthamus tinctorus* accessions. Numbers represent the accessions according to Table 1.

Discussion

A large amount of diversity was found in safflower accessions at agro-morphological and genetic levels. Neither cluster analysis nor PCA revealed a clear relationship between diversity pattern and geographical origin of genotypes and accessions from different origins and were also randomly distributed over the whole dendrogram. For most of the studied morphological traits, similarity among accessions was independent of their origin, and couldn't separate the accessions on the basis of geographical origin. PCA analysis determined that plant height and blooming time are the most important traits which are responsible for variation in our material. Consequently, the most diverse accessions could be selected on the basis of these two principal components. Results of phenotypic measurements were agreement with those obtained by Tuncturk and Ciftci (24). In this study, RAPD markers generated the highest level of polymorphism (65.5%) supported by AFLP (63.1%) markers (Table 1). Based on comparison of information generated with RAPD and AFLP

marker (Tables 1, 8), AFLPs were found to be most informative in discriminating the present safflower accessions. This is also consistent with previous studies where AFLP markers have been able to assign genotypes to known heterotic or other pre defined groups (25).

The concomitant wide range of genetic and morphological similarities (haven't shown) obtained with the three marker systems illustrates a wide genetic base of this crop and the possibility of its improvement through marker assisted breeding programs. The level of polymorphism obtained with AFLP markers were higher than previous reports for other self- and cross-pollinated crops such as Cucumis melo (23.1) (26) but less than reported for *Brassica rapa* (80%), *Vigna angularis* (83%). (27, 28).

Table 8. Comparison of information generated with RAPD and AFLP marker systems in regional accessions. Because of one accession of wild and SYR,%P calculates is zero. N: number of band, H: mean heterozigosity, Na: observed number of alleles, Ne: effective number of alleles, D: gene diversity, I: shannon's information index, P: number of polymorphic loci.

	N	H	Na	Ne	D	I	P	%P
				RAPD				
MEX	57	0.107	1.2692	1.1893	0.1072	0.1572	21	26.92%
CAN	54	0.117	1.2821	1.1994	0.1168	0.1706	22	28.21%
EGY	57	0.133	1.3205	1.2266	0.1328	0.1938	25	32.05%
USA	54	0.149	1.359	1.2538	0.1487	0.2171	28	35.90%
IRN	72	0.294	1.7949	1.5149	0.2941	0.4352	62	79.49%
CIM	52	0.085	1.2051	1.145	0.085	0.124	16	20.51%
SYR	45	0.000	1	1	0	0	0	0
wild	42	0.000	1	1	0	0	0	0
				AFLP				
MEX	178	0.059	1.1513	1.1022	0.0588	0.0867	36	15.13%
CAN	165	0.031	1.0756	1.0535	0.0313	0.0457	18	7.56%
EGY	182	0.091	1.2185	1.1545	0.0905	0.1321	52	21.85%
USA	165	0.037	1.0882	1.0624	0.0365	0.0534	21	8.82%
IRN	188	0.070	1.2059	1.1225	0.0698	0.1045	49	20.59%
CIM	167	0.031	1.0756	1.0535	0.0313	0.0457	18	7.56%
SYR	160	0.000	1	1	0	0	0	0.00%
Wild	161	0.000	1	1	0	0	0	0.00%

Table 9. Partitioning of genetic diversity between and within regional accessions by AMOVA.

Source of variation	DF	SS	Sum of squares	Variance components.	Percentage of variation
			RAPD		
Among regional accessions	5	53.881	10.776	0.000	0%
Within regional accessions	12	148.619	12.385	12.385	100%
Total	17	202.500	23.161	11.806	
			AFLP		
Among regional accessions	5	73.714	14.743	1.204	10%
Within regional accessions	12	136.786	11.399	11.399	90%
Total	17	210.500	26.142	12.603	

There was no association between agro-morphological diversity and molecular diversity. Similar disparity between morphological traits and RAPDs was reported in different studies (29, 30). There could be many reasons for the lack of correlation between RAPDs, AFLP and morphological distances. One reason could be that RAPDs and AFLP detect polymorphisms in coding as well as non-coding regions of the genome (1), of which only a small portion is coding, therefore, it is very likely that the polymorphism found is in a non-coding region. The relationship between molecular markers and phenotypic traits could be significant if the markers were linked to selected loci (31). Also, plants that are morphologically similar are not necessarily genetically (1). Discordance between various DNA marker systems is not uncommon and is reported in many plant taxa (32-35). The incongruence between the marker systems also suggests that RAPD and

AFLP marker systems have different mutation rates u nder similar selective forces in safflowers. According to Powell et al. (36), the relationships may be rather dependent on genome coverage and/or the type of sequence variation recognized by each marker system. More detailed studies are needed for safflowers before any conclusions can be made with regard to genome coverage of markers.

Reference

1. Khan,M.A., Witzke-Ehbrecht,S., Maass,B.L. and Becker,H.C. (2008) Relationships among different geographical groups, agro-morphology, fatty acid composition and RAPD marker diversity in Safflower (Carthamus tinctorius). *Genet. Resour. Crop. Evol.*, **56**, 19–30.
2. Murphy,D. (1999) The future of new and genetically modified oil crops. In Janick,J. (ed), *Perspectives on new crops and new uses.* ASHS Press, Alexandria, Virginia, pp. 216–219.
3. Thies,E. (2000) Promising and Underutilized Species Crops and Breeds Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ) GmbH, Eschborn.
4. Ashri,A., Urie,A.L. and Zimmer,D.E. (1976) Evaluation of the germ plasm collection of safflower, Carthamus tinctorius L. VII. Variability of capitulum width and outer involucral bracts' dimensions. *Euphyt.*, **25**, 225–229.
5. Weiss,E. (1983) Safflower. In *Oilseed crops*. Longman, London, pp. 216– 281.
6. Padulosi,S., Eyzaquirre,P. and Hodgkin,T. (1999) Challenges and strategies in promoting conservation and use of neglected and underutilized crop species. In: Janick J (ed) Perspectives on new crops and new uses. In Janick,J. (ed), *Perspectives on new crops and new uses*. ASHS Press, Alexandria, Virginia, pp. 140–145.
7. Yang,Y.-X., Wu,W., Zheng,Y.-L., Chen,L., Liu,R.-J. and Huang,C.-Y. (2007) Genetic diversity and relationships among safflower (Carthamus tinctorius L.) analyzed by inter-simple sequence repeats (ISSRs). *Genet. Resour. Crop. Evol.*, **54**, 1043–1051.
8. Khan,M. and Witzke-Ehbrecht,S. von (2004) Evaluation of a worldwide collection of safflower for morphological diversity and fatty acid composition. *Vortrage fur Pflanzenzu chtung*, **62**, 104–106.
9. Dwivedi,S.L., Upadhyaya,H.D. and Hegde,D.M. (2005) Development of Core Collection using Geographic Information and Morphological Descriptors in Safflower (Carthamus tinctorius L.) Germplasm. *Genet. Resour. Crop. Evol.*, **52**, 821–830.
10. Jaradat,A.A. and Shahid,M. (2006) Patterns of Phenotypic Variation in a Germplasm Collection of Carthamus tinctorius L. from the Middle East. *Genet. Resour. Crop. Evol.*, **53**, 225–244.
11. Zhang,Z. (2001) Studies on genetic diversity and classification of safflower (Carthamus tinctorius L.) germplasm by isozyme techniques. In Bergman,J., HH.,M. (eds), *Proceedings of the 5th International Safflower Conference*. Williston, ND and Sidney, pp. 157–162.
12. Sehgal,D. and Raina,S.N. (2005) Genotyping safflower (Carthamus tinctorius) cultivars by DNA fingerprints. *Euphyt.*, **146**, 67–76.
13. Yang,Y.-X., Wu,W., Zheng,Y.-L., Chen,L., Liu,R.-J. and Huang,C.-Y. (2007) Genetic diversity and relationships among safflower (Carthamus tinctorius L.) analyzed by inter-simple sequence repeats (ISSRs). *Genet. Resour. Crop. Evol.*, **54**, 1043–1051.
14. Panahi,B. and Ghorbanzadeh Neghab,M. (2013) Genetic characterization of Iranian safflower (Carthamus tinctorius) using inter simple sequence repeats (ISSR) markers. *Physiol. Mol. Biol. Plants*, **19**, 239–243.
15. Mohammadi,S.A. and Prasanna,B.M. (2003) Analysis of Genetic Diversity in Crop Plants—Salient Statistical Tools and Considerations. *Crop Sci.*, **43**, 1235.
16. Rohlf FG. NTsys-pc numerical taxonomy and multivariate system version 2.0 Applied. Biostatistics. Inc, New York, USA.2000; 12:343-348.
17. Vos,P., Hogers,R., Bleeker,M., Reijans,M., Van de Lee,T., Hornes,M., Frijters,A., Pot,J., Peleman,J. and Kuiper,M. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic acids research*, **23**, 4407–14.
18. Sneat,P. and Sokal,R. (1973) Numerical Taxonomy WH Freeman, San Francisco.
19. Mantel,N. (1967) The detection of disease clustering and a generalized regression approach. *Can. Res.*, **27**, 209–220.
20. Belaj,A., Satovic,Z., Cipriani,G., Baldoni,L., Testolin,R., Rallo,L. and Trujillo,I. (2003) Comparative study of the discriminating capacity of RAPD, AFLP and SSR markers and of their effectiveness in establishing genetic relationships in olive. *Theor. Appl. Genet.*, **107**, 736–44.
21. Anderson,J., Churchill,G., Autroque,J., Tanksley,S. and Swells,M. (1993) Optimising selection for plant linkage map. *Genome*, **36**, 181–186.
22. Excoffier,L., Smouse,P. and Quattro,J. (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.
23. Hajghani,M., Saffari,M. and Moud,A.M. (2009) Path coefficient analysis for the yield components of spring safflower cultivars (Carthamus tinctorius L.) in Iran under different nitrogen levels. *American-Eurasian J. Agric. Environ. Sci.,* **6**, 737–740.
24. M.T. and V.C. (2004) Relationships among Traits Using Correlation and Path Coefficient Analysis in Safflower (Carthamus tinctorius L.) Sown Different Fertilization Levels and Row Spacing. *Asian J. of Plant Sci.*, **3**, 683–686.
25. Soleimani,V.D., Baum,B.R. and Johnson,D.A. (2002) AFLP and pedigree-based genetic diversity estimates in modern

cultivars of durum wheat [Triticum turgidum L. subsp. durum (Desf.) Husn.]. *Theor. App. Genet.*, **104**, 350–357.

26. Bohn,M., Utz,H. and Melchinger,A. (1999) Genetic similarities among winter wheat cultivars determined on the basis of RFLPs, AFLPs, and SSRs and their use for predicting progeny variance. *Crop sci.*, **39**, 228–237.

27. Negi,M.S., Sabharwal,V., Bhat,S.R. and Lakshmikumaran,M. (2004) Utility of AFLP markers for the assessment of genetic diversity within Brassica nigra germplasm. *Plant Breed.*, **123**, 13–16.

28. Zhao,J., Wang,X., Deng,B., Lou,P., Wu,J., Sun,R., Xu,Z., Vromans,J., Koornneef,M. and Bonnema,G. (2005) Genetic relationships within Brassica rapa as inferred from AFLP fingerprints. *Theor. Appl. Genet.*, **110**, 1301–14.

29. Vollmann,J., Grausgruber,H., Stift,G., Dryzhyruk,V. and Lelley,T. (2005) Genetic diversity in camelina germplasm as revealed by seed quality characteristics and RAPD polymorphism. *Plant Breed.*, **124**, 446–453.

30. Johnson,R.C., Kisha,T.J. and Evans,M.A. (2007) Characterizing Safflower Germplasm with AFLP Molecular Markers. *Crop Science*, **47**, 1728.

31. Persson,H.A. and Gustavsson,B.A. (2001) The extent of clonality and genetic diversity in lingonberry (Vaccinium vitis-idaea L.) revealed by RAPDs and leaf-shape analysis. *Mol. ecol.*, **10**, 1385–97.

32. Staub,J., Danin-Poleg,Y., Fazio,G. and Horejsi,T. (2000) Comparative analysis of cultivated melon groups (Cucumis melo L.) using random amplified polymorphic DNA and simple sequence repeat markers. *Euphytica*, **115**, 225–241.

33. Virk,P.S., Zhu,J., Newbury,H.J., Bryan,G.J., Jackson,M.T. and Ford-Lloyd,B.V. (2000) Effectiveness of different classes of molecular marker for classifying and revealing variation in rice (Oryza sativa) germplasm. *Euphyt*, **112**, 275–284.

34. Ude,G., Pillay,M., Ogundiwin,E. and Tenkouano,A. (2003) Genetic diversity in an African plantain core collection using AFLP and RAPD markers. *Theor. Appl. Genet.*, **107**, 248–55.

35. Souframanien,J. and Gopalakrishna,T. (2004) A comparative analysis of genetic diversity in blackgram genotypes using RAPD and ISSR markers. *Theor. Appl. Genet.*, **109**, 1687–93.

36. Powell,W., Morgante,M., Andre,C., Hanafey,M., Vogel,J., Tingey,S. and Rafalski,A. (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Mol. Breed.*, **2**, 225–238.