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Genetic Diversity Analysis of Safflower (*Carthamus tinctorius*) Accessions from Different Geographic Origins using ISSR Markers

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Abstract

Safflower (*Carthamus tinctorius*) is an oilseed crop with high quality vegetable seed oil. A set of 55 accessions from different geographical origins were introduced to constitute a breeding program germplasm. These materials were characterized by inter-simple sequence repeat (ISSR) as a simple method to reveal polymorphism among them. ISSR analysis was carried out with 13 primers. After agarose gels analysis, ISSR banding patterns were converted into binary data of presence-non presence and matrices were processed. Genetic distances between all pairwise combinations of the accessions were calculated using similarity of simple coefficient matching (SCM). Dendrogram of studied accessions was constructed using the UPGMA (unweighted pair group method arithmetic average) method. A high number of reproducible ISSR bands exhibited a high percentage of polymorphism (69.64%), indicating a high genetic diversity among the material studied. The lowest similarity coefficient was 0.462, observed between accessions 109 and 135, whilst the highest one was 0.962, observed between accessions 68 and 126, with an average of 0.726. Dendrogram similarity relationships revealed some clusters according to different genetic pools. The ISSR markers were very informative and exhibited a high level of polymorphism. The findings of this study would be useful for safflower breeding program in Morocco as well as in other countries of the world.

Keywords: Safflower; ISSRs markers; Genetic diversity; Polymorphism; Breeding program

Introduction

Safflower (*Carthamus tinctorius* L.) belongs to the Asteraceae family. It is one of the remarkable oldest domesticated crops (Hamdan *et al.*, 2011). It has been planted since ancient times both as a dyestuff and as oil crop in a wide range of geographical regions (Knowles, 1989). It is cultivated in some regions of Europe, Australia, Asia, and the Americas to produce high quality vegetable and industrial oil (Sehgal *et al.*, 2009). Nowadays, it is cultivated mainly for its seed, which is used as source of edible oil and as birdseed (Dajue and Mündel, 1996).

Safflower is a minor crop and in 2013, just 22 countries grew it (FAOSTAT, 2013). In that year, 782,641 hectares were cultivated worldwide, ensuring a production of about 647,374 tons. India is the major producer with 109,000 tons produced on 150,000 hectares (FAOSTAT, 2013). In the last few years, the safflower has been used as a biofuel, pharmaceutical production (McPherson *et al.*, 2004) and particular oil types (Velasco and Fernandez-Martinez, 2004). Thereby, the interest in this crop has been increasing by both private and public sectors. Safflower has a good yield potential in Morocco as well as in other semiarid areas across the world. Safflower is suggested to be a winter crop in various zones of Morocco. These include low rainfall regions where the production of other oilseed crops is scarce (Zraibi *et al.*, 2012). Recent and future investigations about safflower may lead to the growth of this crop as important oilseed crop (Nabloussi and Boujghagh, 2006). In this context, a new safflower-breeding program has been recently launched. Many accessions from different geographic origins were provided by the USDA-ARS of Washington (USA) to constitute the initial germplasm. Genetic diversity is the raw material to release varieties adapted to different environments with better yield (Handaji *et al.*, 2012).

Biochemical, morphological and molecular markers are exploited to evaluate plant genetic diversity. During the past decades, molecular breeding, which has involved both markers assisted selection and genetic engineering, has witnessed significant innovations and advances (Xu *et al.*, 2012). Among various genetic markers, it is very important to identify, characterize and select the most appropriate ones for effective molecular studies. The most useful molecular markers are inter-simple sequence repeats (ISSR), random amplified polymorphic DNA (RAPD) and amplified

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fragment length polymorphism (AFLP) (Kumari *et al.*, 2013). These previous markers are used for crops with inadequate genomic resources because they do not need prior sequence information, and they have been used especially for assessing species genetic diversity (Yazdi-Samadi *et al.*, 2001; Sehgal and Raina, 2005; Johnson *et al.*, 2007). However, microsatellite markers are specific markers that require prior development for each species (De Vienne, 1998). Inter-simple sequence repeat (ISSR) is a DNA based marker with primers based upon dinucleotide, tetra or pentanucleotide repeats. The ISSR markers, being simple, acceptably stable and highly reproducible, have been swimmingly used in genetic variation studies. These markers are a convenient option for providing preliminary genotyping data for a species without the need for prior genomic DNA sequence information (Chen *et al.*, 2014). This information makes selection of elite genotypes easy for safflower breeding programs (Kumar *et al.*, 2015).

To the best of our acquaintance, no ISSR characterization of the international safflower collection provided and used in this study has been carried out. Therefore, the objective of this research was the molecular characterization of the genetic diversity among accessions of this collection, using the ISSR markers, and the identification of homogeneous genetic pools from this collection to be exploited and used in breeding program of this species.

Materials and Methods

Plant Materials

This study concerned 55 genotypes consisting of 54 accessions from different geographic origins and provided by the United States Department of Agriculture-Agricultural Research Service, and one cultivar provided by CSIC, IAS-Cordoba (Spain) namely 'Rancho', used as a check. Table 1 shows the name/code, and the origin of these genotypes.

DNA Isolation

Genomic DNA was isolated from young leaves (21–28 days old seedling) of plants from each genotype following the Cetyl Trimethyl Ammonium Bromide (CTAB) modified method of (Saghai Maroof *et al.*, 1984).

Amplification of the DNA by ISSR Markers

ISSR based PCR was carried out as mentioned by Sbabou *et al.* (2010), using 13 ISSR primers. Sequences of these primers were selected after referring to the literature. Gradient PCR was used to adjust the annealing temperature of each primer. Primer sequences and melting temperatures are shown in Table 2. DNA was amplified by PCR in a total volume of 25 µL containing 15 ng/µL of DNA, 5 µL of PCR tampon (110), 50 mM of MgCl2, 2.5 mM of dNTP, 10 pmol/µL of each ISSR primer, and 2 units DNA polymerase (PROMEGA). The amplification was executed in the Eppendorf Master Cycler gradient. PCR was optimized according to the following amplification program: Denaturation step at 94°C for 30 s followed by 40 cycles with a denaturation step at 94°C for 1 min, hybridization at 45°C for 1 min and an elongation step at 72°C for 2 min. Amplification is sealed off by an elongation step at 72°C for 7 min. The ISSR amplification products were separated by gel electrophoresis on a 2% agarose gel, in Tris Borate EDTA (TBE) buffer during 2.5 h at 150 Volt and stained with ethidium bromide (100 mg/mL). The ultraviolet (UV) images of gels are photographed and stored on computer for subsequent treatment by Mesurim software.

Data Scoring

The amplified products for ISSR marker loci at a specific position in a gel were scored visually as ''1'' for the presence and ''0'' for the non-presence of a band to generate a binary data matrix. Only net strips obtained, also called markers are detected.

Data Analysis

From data created through binary scoring (presence/absence) of the gel photo, genetic distance and similarity were calculated using simple coefficient matching (SCM) by Clustering Calculator software program established by Brzustowski (2002). Based on genotypes pairwise comparison, a histogram according to the bands number that distinguishes the pairs has been established. On the other hand, for measuring the ISSR informativeness to differentiate the 55 safflower genotypes, the polymorphic information content (PIC) was calculated using the formula of Anderson *et al.* (1993): PIC = 2fi (1-fi), where fi is the percentage of the amplified band 'i'. Thus, for each primer, PIC value was calculated as the average of all PIC markers (bands). The Cluster analysis was done to group the genotypes into a dendrogram. The dendrogram was constructed on the basis of the similarity matrix data using the unweighted pair group method, arithmetic averages (UPGMA) and numerical taxonomy system (NTSYS-pc) software (Rohlf *et al.*, 2002; Alam *et al.*, 2015).

Results

Polymorphism

Among the 13 ISSR primers tested, six primers (46.15%) [F4, F8, IMA834-1, 1MA5-1, UBC856 and UBC818X] have generated non-readable amplification profiles (Table 3). Seven primers (53.84%) have shown readable amplification profiles with an interesting number of bands (Fig. 1). The number of polymorphic products ranged from four, for F3 and F1, to nine for F9. Indeed, 56 ISSR bands were recorded with 39 polymorphic fragments (69.64%). The size of the observed bands varied between 190 and 2845 bp (Table 3).

Table 1: Identification of the 55 safflower genotypes used in the present study

Code INRA	Code/name	Origin	Code INRA	Code/name	Country of origin
1	PI 286199	Kuwait	113	PI 250523	Egypt
2	PI 262430	Syria	114	PI 250527	Egypt
3	PI 237549	Sudan	116	PI 250077	Egypt
4	PI 209295	Kenya	117	PI 250528	Egypt
8	PI 251979	Turkey	118	PI 250537	Egypt
10	PI 253391	Spain	119	PI 250538	Egypt
31	PI 306684	Unknown	120	PI 250539	Egypt
32	PI 262431	Unknown	122	PI 306612	Egypt
35	PI 248631	Pakistan	124	PI 306609	Egypt
52	PI 304592	Afghanistan	126	PI 306607	Egypt
68	PI 613498	USA	132	PI 306605	Egypt
69	PI 537599	USA	133	PI 306602	Egypt
75	PI 560172	USA	135	PI 306600	Egypt
79	PI 560161	USA	136	PI 306598	Egypt
84	PI 307060	India	137	PI 306596	Egypt
86	PI 306928	India	138	PI 250920	Iran
90	PI 307085	India	150	PI 250823	Iran
91	PI 248381	India	151	PI 250821	Iran
94	PI 306847	India	152	PI 250820	Iran
98	PI 305212	India	155	PI 222240	Iran
99	PI 248382	India	157	PI 304467	Iran
102	PI 248362	India	158	PI 250827	Iran
106	PI 250609	Egypt	162	PI 304471	Iran
107	PI 250610	Egypt	163	PI 304472	Iran
109	PI 250605	Egypt	164	PI 304476	Iran
110	PI 250540	Egypt	165	PI 304462	Iran
111	PI 250526	Egypt	Rancho	Rancho	Spain
112	PI 250079	Egypt			

Table 2: List of 13 different ISSR primers and annealing temperatures used in the genetic characterization of 55 safflower genotypes

Primers	Sequences (5'-3')	Annealing temperature(°C)
F1	$(CA)_6$ AT	48°C
F2	$(CA)_6$ GC	53°C
F3	$(CA)_6$ AG	53°C
F4	$(AGC)_4$ CT	50°C
F7	$(AC)_8$ CT	53°C
F8	$(AG)_8$ CC	50°C
F9	$(AG)_8$ CG	53°C
F10	$(CA)_8$ AG	56°C
IMA 12-4	$(CA)_8$ TC	48°C
IMA 834-1	$(AG)_8$ YT	45°C
1MA5-1	$(CA)_8$ TG	57°C
UBC856	$(GGAGA)_3$	56°C
UBC818X	$(CA)_8$ G	55°C

The obtained results showed that all primers reproducible produced polymorphic bands, except IMA 12-4 primer with no diversity, having a single monomorphic marker. Maximum diversity was observed for the primer F9 (Fig. 1) exhibiting 90% of polymorphic bands (Table 3). Average of polymorphic bands per primer was 63.38%. One could observe that the most important polymorphic bands was related to (CA)n, (AC)n and (AG)n primers.

Classification of Accessions by ISSR

Genetic similarity

Similarities between the different accessions were calculated on the basis of the obtained 56 ISSR markers (Table 4). They were very significant and deducted according to the observed percentages. Similarity coefficient varied from 0.462 to 0.962, with an average of 0.726. The highest similarity coefficient (0.962) was recorded between the accessions 68 and 126, indicating that they have the same appearance. Only two dissimilar markers differentiated both genotypes (Table 4). The lowest similarity coefficient (0.462) was detected between the accessions 135 and 109, suggesting maximum genetic distance between both accessions. Twenty-eight dissimilar markers differentiated these genotypes (Table 4).

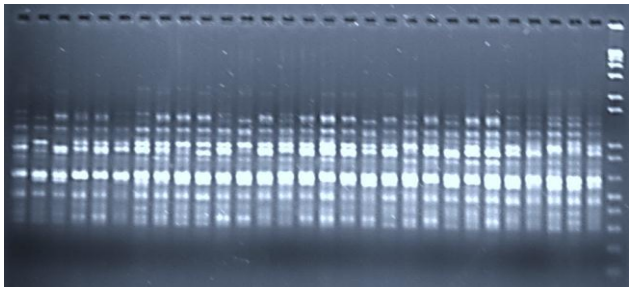

Fig. 1: PCR amplification profile of ISSR marker F9 in safflower genotypes

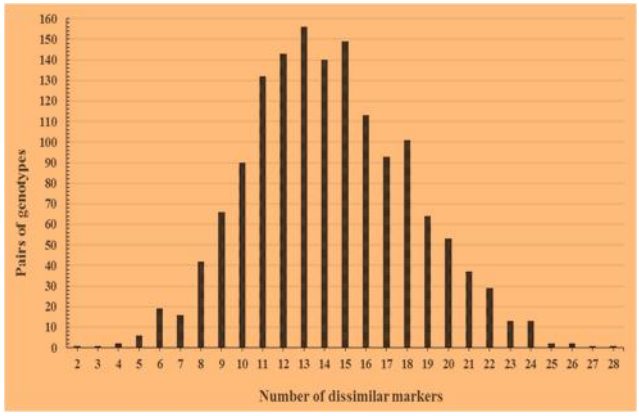

Fig. 2: Frequency distribution of genetic dissimilarity for all pairwise combinations among 55 safflower genotypes

Among the 1485 pairwise combinations, only 45 ISSR profile pairs were differentiated by less than 7 markers. The other 1440 pairs were distinguished by 8 to 28 markers (Fig. 2).

Polymorphism Information Content (PIC)

Seven primers generated polymorphisms with variable and significant genetic diversity index (PIC). The more its value tends towards one more the primer in question is polymorphic. For the seven primers, PIC values ranged from 0 to 0.40 with an average of 0.23. The highest value was recorded for the primer F9, confirming the highest diversity in this primer. The primer F2 recorded the lowest PIC value, indicating the lowest diversity in this primer.

Table 3: ISSR primers, number of fragments, number of polymorphic fragments, polymorphic information content (PIC) and percentage of polymorphism generated in safflower genotypes characterization

Primers	Range of the band size (bp)	Polymorphism (%)	Number of polymorphic bands	Number of monomorphic bands	PIC
F1	250-2153	66.66	4	2	0.22
F2	190-2058	80	8	2	0.13
F3	336-2250	67	4	2	0.34
F4	-	-	-	-	-
F7	318-2240	70	7	3	0.32
F8	-	-	-	-	-
F9	217-2168	90	9	1	0.40
F10	235-2845	70	7	3	0.21
IMA12-4	538-1002	0	0	4	0
IMA834-1	-	-	-	-	-
1MA5-1	-	-	-	-	-
UBC856	-	-	-	-	-
UBC818X	-	-	-	-	-
AverageTotal		63.38%	39	17	0.23

For the primer IMA 12-4, the PIC value is zero due to the absence of polymorphic bands, confirming that primer had no diversity.

Cluster Analysis

From the obtained dendrogram, the 55 safflower accessions were grouped into four major clusters and nine independent branches. Three of these branches originated from Iran (157,158,162), Three from India (90, 94, and 99), two from Egypt (135,109), and only one from Sudan (3). The groups' similarity coefficient varied from 0.671 to 0.866. According to Fig. 3 and Table 5, the following accessions: 110, 111, 106, 107, 119, 112, 116, 133, 137, 114, 118, 117, 120, 136 and 132 from Egypt, 152, 151, 155, 138, 150, 122 and 124 from Iran, and one accession whose origin is unknown, have clustered in a same group with 23 accessions (Cluster III). The group corresponding to Cluster II was constituted by 15 accessions from various geographical origins. The smallest Clusters were I and IV containing, each, four different accessions. The check variety 'Rancho' was found in the Cluster IV with three Iranian accessions, 163, 164 and 165.

Discussion

The markers described in this paper represent a valuable resource for safflower genetic analysis. The high number of polymorphic bands, which ranged from four to nine, implies that ISSR markers could detect variation at DNA level for the studied plant materials. Primers, which did not amplify DNA of all genotypes, might not have found complementary sequences on the genomic DNA. Such non-amplifying primers were also reported in other crop plants (Bhagyawant and Srivastava, 2008). Also, the high percentage of polymorphic bands per primer (63.38%) indicates and reflects the high level of polymorphism revealed by the selected primers among the studied accessions. In a study of safflower wild relatives, using nine ISSR primers, Yaman *et al.* (2014) found that there was significant diversity among 39 species native to several cities in Turkey. This was indicated by the high number of obtained polymorphic bands (151 bands), with an average of 16 bands per primer. However, this very high number of polymorphic bands was a result of inter-species characterization. Similarly to our study, previous intra-species studies have shown a high level of polymorphism detected by ISSR markers: In Chinese barley accessions (98.13%) (Hou *et al.*, 2005), in Portuguese bread wheat cultivars (98.5%) (Carvalho *et al.*, 2010) and in Mediterranean faba bean cultivars (98.9%) (Terzopoulos and Bebeli, 2008). Obtaining a high number of polymorphic ISSR primers is greatly useful. It increases the reliability of

Table 4: ISSR similarity coefficients recorded between safflower accessions and corresponding numbers of pairs of genotypes and dissimilar markers

Similarity	Pairs of genotypes number	Number of dissimilar markers
0.962	1	2
0.942	1	3
0.923	2	4
0.904	6	5
0.885	19	6
0.865	16	7
0.846	42	8
0.827	66	9
0.808	90	10
0.788	132	11
0.769	143	12
0.750	156	13
0.731	140	14
0.712	149	15
0.692	113	16
0.673	93	17
0.654	101	18
0.635	64	19
0.615	53	20
0.596	37	21
0.577	29	22
0.558	13	23
0.538	13	24
0.519	2	25
0.500	2	26
0.481	1	27
0.462	1	28
Total	1485	

Table 5: Accessions clustering obtained from ISSR-based dengrogram using UPGMA method

Clusters	Accessions	Origin, and number of accessions by country	Total number of accessions
Cluster I	1, 10, 32, 2	Kuwait (1), Syria(1) Spain(1),Unknown(1)	4
Cluster II	4, 68, 126, 69, 113, 84, 52, 75, 79, 86, 91, 98, 102, 8, 35	Egypt (2), Kenya (1), Turkey (1), Pakistan (1), Afghanistan (1) USA (4), India (5)	15
Cluster III	31, 110, 111, 106, 107, 119, 112, 116, 133, 137, 114, 118, 117, 120, 136, 132, 152, 151, 155, 138, 150, 122, 124	Egypt (17), Iran (5) Unknown(1)	23
Cluster IV	163, 164, 165, Rancho	Iran (3), Spain (1)	4
Branches	90, 135, 157, 158, 162, 94, 99, 109, 3	Egypt (2), India (3) Iran (3), Sudan(1)	9

the results interpretation, especially when the generated profiles are reproducible. Number of polymorphic primers used to analyze various plant species depends upon this species and differs from one study to another. It was seven in silkworm (Pradeep *et al.*, 2005), 10 in pistachio (Kebour *et al.*, 2012), 12 in basmati (Nagaraju *et al.*, 2002), 15 in lupine (Sbabou *et al.*, 2010), 23 in *Asparagus acutifolius* (Sica *et al.*, 2005), 30 in *Lupinus spp*. (Talhinhas *et al.*, 2003) and 41 in sorghum (Yang *et al.*, 1996). In our research, the most polymorphic bands was associated to (CA)n, (AC)n, and (AG)n primers. This is in agreement with the findings of a previous study on safflower having also indicated that (AG)n and (CA)n ISSR primers created the highest polymorphic bands (Golkar *et al.*, 2011). These motifs might have a significant contribution in the safflower genome. Generally, in plants, polymorphic information content (PIC) values vary from zero, for monomorphic markers, to 0.5 for polymorphic markers (Roldan-Ruiz *et al.*, 2000). In our study, PIC values varied from 0.13 to 0.40, indicating that ISSR markers are very informative, which can be examined to correlate banding patterns and agronomic characteristics. This result is in agreement with that of Tonk *et al.* (2014) who found value ranging from 0.12 to 0.49 when they characterized triticale genotypes using ISSR primers. Farsani *et al.* (2012) found PIC values ranging from 0.26 to 0.46 in the characterization of Bermudagrass (Cynodon dactylon). Alam *et al.* (2015) reported values varying from 0.22 to 0.37 in their molecular study on purslane (*Portulaca oleracea* L.) using ISSR markers.

Similarity coefficient between the studied accessions varied from 0.462 to 0.962, with an average of 0.726. Kebour *et al.* (2012) found similar results when they studied genetic diversity and relationship among 10 *Pistacia vera* L. cultivars using 10 ISSR markers. The average similarity across all the genotypes showed there was considerable genetic diversity among the safflower genotypes examined. Overall, accessions structuring and hierarchical classification of the obtained groups highlight the degree of diversity among the accessions studied. This diversity has been confirmed by pairwise comparison of the accessions. In fact, most of these genotypes were differentiated by more than eight markers. The small differences observed (less than 7 markers) were probably due to gel reading problem and technical nature of ISSR markers.

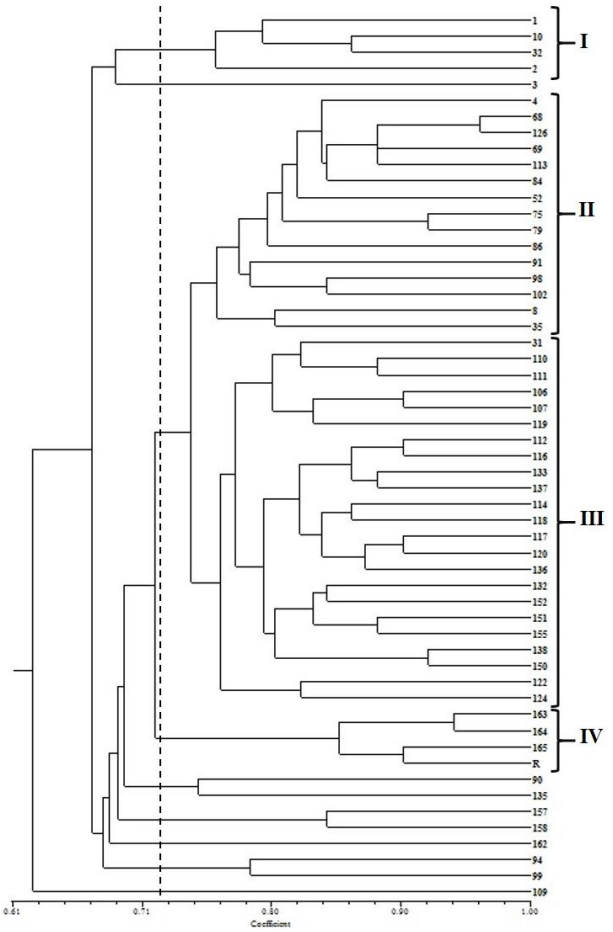

Fig. 3: Dendrogram generated for 55 safflower genotypes using UPGMA cluster analysis

The cluster analysis performed by ISSR data showed there was some correlation between the geographical origin and the genomic similarities. In a previous study, based on ISSR markers, similar findings were reported for safflower genetic variation based on seed quality-related traits (Arzani and Rezaei, 2011). In molecular clustering point of view, similarities in genotypes grouped in the same cluster could also arise because of sharing a common parentage, convergent evolution and selection. The Iranian accessions, 163, 164 and 165 were, genetically, the closer to the Spanish variety 'Rancho'. Accessions having clustered in the same

group could have a common ancestor or they could be due to plant material exchange between countries. Yang *et al.* (2007) had characterized 48 safflower accessions using ISSR markers and had reported similar conclusion when they had observed that accessions originating from Europe were relatively grouped together. These authors had also showed that the 48 accessions could be divided into nine groups by using UPGMA method. Panahi and Ghorbanzadeh-Neghab (2013) observed two main clusters and four subclusters in the ISSR molecular characterization of 18 accessions of Iranian safflower. Wodajo *et al.* (2015) showed that dispersion into various groups of Ethiopian safflower using ISSR markers appeared to be at random. However, few accessions formed distinct clusters based on their geographic origins.

According to findings of this research, we can deduce that ISSR primers were informative markers and allowed us to detect considerable polymorphism in our genetic materials, suggesting that they could be very useful in safflower germplasm characterization and fingerprinting purposes. The genetic diversity study is very important for the choice of parents to be crossed in breeding program (Alam *et al.*, 2015). An effective germplasm assessment provides scientific basis for breeding safflower for specific agro-ecological conditions. The results obtained with the help of ISSR markers will be confronted with the ongoing morphological and agronomic evaluation of the accessions investigated in the present work.

Conclusion

Similarity coefficient and PIC values found in this study indicated that ISSR markers were very informative and there was considerable genetic diversity among the assessed accessions. This diversity has been confirmed by pairwise comparison of the accessions. Most of these genotypes were differentiated by more than eight markers. Therefore, ISSR markers used in the present study allowed us to reveal an important genetic diversity among the characterized safflower accessions. These markers provide interesting tools for safflower conservation and utilization for seed bank management and breeding purposes. The 55 safflower accessions were grouped into four major clusters and nine independent branches. The cluster analysis revealed there was some correlation between the geographical origin and the genomic similarities. The clustering groups obtained should be confronted to the groups to be observed on the basis of ongoing morphological, agronomic and physiological evaluation of these accessions. The confirmed homogeneous groups will be multiplied, conserved and then could be used as different genetic pools in safflower breeding program in Morocco as well as in other safflower cultivation areas in the world.

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