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Hamed Bagmohammadi[1], Mohammadhadi Pahlevani[1],
Asadollah Ahmadikhah[2], Seyed Esmaeil Razavi[1]

[1]Department of Plant Breeding and Biotechnology, College of Plant Production, Gorgan University of Agricultural Sciences and Natural Resources, P.O. Box 386, Gorgan, Iran; [2]Department of Agronomy and Plant Breeding, Faculty of Agriculture, Zanjan University, Zanjan, Iran

GENETIC VARIATION OF SAFFLOWER (*CARTHAMUS TINCTORIUS* L.) AND RELATED SPECIES REVEALED BY ISSR ANALYSIS

ABSTRACT

Genetic diversity of eight genotypes of *Carthamus tinctorius* L., two populations of *C. oxyacanthus*, and one population of *C. lanatus* was investigated using inter-simple sequence repeat (ISSR) markers. All samples were uniquely distinguished by 10 ISSR primers with 144 bands which generated 100% polymorphism. Furthermore, the ISSR markers could separate three safflower species properly, that highlights the effectiveness of this marker system for phylogenetic studies. The most and least informative primers were ISSR9 (PIC=0.367) and ISSR2 (PIC=0.254), and some primers were more efficient in detecting polymorphism in one species than for the others. Unweighed pair-group method with arithmetical averages (UPGMA) cluster analysis enabled construction of a dendrogram for estimating genetic distances among different populations. The result of cluster analysis suggested that cultivated and wild populations of *C. oxyacanthus* had close relationship with each other and far relationship with *C. lanatus*. The extreme genetic dissimilarity was observed between genotypes of *C. tinctorius* and *C. lanatus* populations. Based on the results, *C. oxyacanthus* could introduce favorable genes to cultivated safflower via inter-specific hybridization in breeding programs. Nei's gene diversity index, Shannon's index and percent of polymorphic loci showed that Isfahan ecotype of C. *oxyacanthus* had the highest variation at DNA level in relation to populations of other species. The ISSRs developed in this research along with those recently studied by other researchers will contribute to construct genetic map with a density sufficient for safflower molecular breeding.

Key words: Genetic diversity, ISSR markers, relationships, safflower, wild spices

INTRODUCTION

The genus *Carthamus* from the family *Asteraceae* contains about 25 species native to the Mediterranean region and Asia. In Iran three species of

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C. tinctorius, *C. oxyacanthus* and *C. lanatus* are more widespread (Zeinali, 1999). Most of the *Carthamus* species are classified as noxious weeds. Safflower, *Carthamus tinctorius* (2n=2x=24), is the only cultivated species of the genus. Safflower, as an economically important member, was growing as a natural dye source for dye making and for medicinal purposes, but it is now grown worldwide as a source of high quality vegetable and industrial oil. *C. oxyacanthus* (2n=2x=24), one of the important wild species, is widespread from western Iraq, Iran, Afghanistan to north of India (Dajue and Mundel, 1996). In addition, the oil content and quality of *C. oxyacanthus* are comparable to those of the cultivated genotypes (Sabzalian *et al.*, 2008). *C. lanatus* (2n=2x=44) also, is a weed of the roadside and of range areas (Knowles and Ashri, 1958). It is also assumed that cultivated safflower may origins from *C. oxyacanthus* and *C. lanatus* (Chavan, 1961). These two species have an important role in breeding programs and for improvement of new safflower genotypes with favorable traits such as resistance against some diseases, pest, cold and drought (Heaton and Klisiewicz, 1981; Sabzalian *et al.*, 2010; Majidi *et al.,* 2011). Researchers reported that hybridization with several wild species of *Carthamus* may have played a role in the evolution of *C. tinctorius* in the Mediterranean and Asia where they are sympatric (Ashri and Knowles, 1960). Natural hybrids of *C. tinctorius* and *C. oxyacanthus* have been documented in Iran, Pakistan and India (Deshpande, 1952; Knowles, 1969; Knowles and Ashri, 1995). Heaton and Klisiewicz (1981) obtained hybrids from a cross of *C. tinctorius* and *C. lanatus* when either species was used as the female recipient. They treated the rescued embryos with colchicine causing a doubling of the chromosome number and producing an autopolyploid (n = 34) (Heaton and Klisiewicz, 1981). These researchers also mentioned that further manipulations were required to obtain fertile hybrid plants from these crosses. Thus, the likelihood of a hybrid between cultivated safflower and *C. lanatus* surviving under natural conditions is highly unlikely.

Safflower is underutilized when compared with other oilseed crops like soybean, rapeseed and sunflower, primarily because of low oil content and yield as well as susceptibility to several diseases and insect pest that limit its productivity (Zeinali, 1999). Breeding programs in safflower will be successful only if there is enough variation for agronomically important traits in the germplasm. Landraces of *C. tinctorius* and the wild species of the genus *Carthamus* could provide valuable genes that have been lost during the domestication and help to broaden the safflower gene pool. For example the cultivated species was highly susceptible to *Alternaria carthami*, while the two wild species, *C. lanatus* and *C. oxyacanthus* were immune and tolerant (Prasad and Anjani, 2005). A number of recent studies have addressed this opportunity by investigating genetic variation in several wild species, including *C. lanatus* and *C. oxyacanthus* as well as *C. tinctorius*

landraces from Iran (Maali-Amiri *et al.,* 2001; Amini *et al.*, 2008, Sabzalian *et al.*, 2008).

Breeding programs to improve economic traits have been hampered by limited knowledge about genetic variability within *C. tinctorius* and the lack of efficient genomic tools. In recent years a number of research groups have recognized the need for a molecular characterization of the safflower genome and the development of DNA markers (Maali-Amiri *et al.*, 2001; Sehgal *et al.*, 2009a; Johnson *et al.*, 2007). One of the most important components required for an efficient system of molecular breeding is the identification and characterization of suitable genetic markers. Most molecular markers have used in safflower are random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSRs) and amplified fragment length polymorphism (AFLP). These are usually the markers of choice for crops with inadequate genomic resources, because they do not require prior sequence information, and they have been used mainly for assessing genetic diversity within a species. Maali-Amiri *et al.* (2001) used RAPD markers to find association between genetic diversity and geographic distribution of safflower in Iran. Simple sequence repeats (SSRs) or microsatellite markers consist of repeats of simple nucleotide motifs that are abundant in most eukaryotic genomes. Because of their high degree of polymorphism, codominant Mendelian inheritance, reproducibility and locus-specificity, they have been the markers of choice for genetic mapping and other genomic applications in numerous plant species (Philips and Vasil, 2001). Recently, a number of research groups have recognized the need for SSR development in safflower as a valuable tool for molecular breeding. In this sense, Chapman *et al.* (2009) have generated a set of 104 safflower SSRs from an expressed sequence tag (EST) collection, some of them used for population genetic analysis in this species (Chapman *et al.*, 2010). Mayerhofer *et al.* (2010) have reported the development of the highest collection (more than 1000) of SSRs in safflower to date and have initiated the first major linkage analysis in this species. SSRs are ubiquitous in eukaryotes but require knowledge of flanking sequences to design polymerase chain reaction (PCR) primers for their amplification. ISSR PCR, on the other hand, requires no sequence information and is carried out using single primers based on a simple repeat with the addition of a degenerate nucleotide or a selective anchor. These primers amplify the region between microsatellites, the ISSR region. They rely on the presence of palindromic microsatellites within amplifiable distances. Genetic diversity using ISSR markers have been investigated in world safflower germplasm (Seghal *et al*, 2009), *C. lanatus* populations (Ash *et al.*, 2003) and *C. oxyacanthus* (Sabzalian *et al.*, 2009b).

Evaluation of the genetic diversity in crop species is an essential step for practical applications in plant breeding; such as selection, reduce crossing

costs and parent selection in crossing programs. There are several methods for assessing genetic diversity, but DNA markers have provided valuable tools in the various genetic diversity and relationships analysis. Furthermore, molecular approaches for safflower breeding are very limited. Thus, the goals of this study were (i) to evaluate degree of inter and intra species variation within the genus *Carthamus* by using ISSR analysis, with the purpose of using the wild relatives for safflower breeding, (ii) to recognize a collection of genomic ISSRs as a valuable tool for molecular breeding of safflower and (iii) to assess the informativeness of ISSR markers for revealing inter and intra species differentiation in the genus *Carthamus*.

MATERIALS AND METHODS

Plant materials and DNA extraction

This research was performed at Gorgan University of Agricultural Sciences and Natural Resources (GUASNAR), Gorgan, Iran in 2011. The study was carried out from eight genotypes of *C. tinctorius* and two populations of *C. oxyacanthus* collected from provinces Tehran and Isfahan and one population of *C. lanatus* from Golestan province in Iran (see Table 1). The cultivated genotypes and wild populations were grown for one year before sampling at Research Farm of GUASNAR. DNA of the plant materials was extracted using cetyltrimethylammonium bromide (CTAB) procedure with some modifications (Hulbert and Bennetzen, 1991). For the cultivated genotypes, bulked genomic DNA was extracted from 50 mg of leaf tissue from the first fully expanded leaf of the plants. For the wild populations, DNA was extracted from 50 mg of leaf tissue from single plant samples. Totally 30 single plants of wild populations were sampled for DNA extraction in the wild ecotypes (Table 1). For each sample, 5-10 young leaves were grounded to fine powder in liquid nitrogen and then total DNA was isolated according to the protocol. Then DNA was quantified electrophoretically using lambda standard DNA on 1% (w/v) agarose gels (BioRad, Canada). The extracted DNA was suspended in 100 µl of sterile, distilled water and stored at -20ºC until use. Ten random ISSR primers, 14–16 core nucleotides in length with 2 base anchore, were used for PCR reaction and ISSR analysis (Table 2; CinnaGen, Iran).

The PCR reaction was performed in a 15 µl volumes using a PCR thermocycler (Peqlab Co.). The reaction mixture contained 1µl DNA, 5µl H2O, 0.75 µM × l^{-1} primer and 7.5 µl Master Mix (CinnaGen, Iran). Thermocycler was programmed to 1 cycle of 5 min at 94°C (early denaturation); followed by 35 cycles of 50 sec at 94°C (denaturation), 40 sec at 48°C (annealing), and 1 min at 72°C (extension), ending with 1 cycle of 5 min at

72°C (final extension). PCR amplified products were separated by vertical electrophoresis in 6% polyacrilamide gels (APPELEX, France) and subsequent were stained with silver nitrate. Pictures of DNA banding patterns were taken by VilBer Laurmat Gel Documentation (France).

Table 1

Name and origin of some genotypes and ecotypes of *Carthamus* for ISSR analysis

Genotype	Species	Origin
Arak2811	*C. tinctorius*	Iran
LRV-5151	*C. tinctorius*	Iran
Aceteria	*C. tinctorius*	Canada
Dinger	*C. tinctorius*	Unknown
34074	*C. tinctorius*	Unknown
34040	*C. tinctorius*	Unknown
Pi-50537	*C. tinctorius*	Unknown
541-5	*C. tinctorius*	Unknown
Ecotype	*C. lanatus*	Gloestan province, Iran
Ecotype, Isfahan	*C. oxyacanthus*	Isfahan province, Iran
Ecotype, Tehran	*C. oxyacanthus*	Tehran province, Iran

ISSR analysis

Amplified products were scored by the presence (1) or absence (0) of the bands. Achieved binary matrix was used to compute genetic similarity coefficient (GS) according this function (Nei and Li, 1979):

$$GS = \frac{2 \times N_{xy}}{n_x + n_y}$$

where, N_{xy} is the number of shared band between two genotypes, n_x is total band number for genotype x; and n_y is total band number for genotype y. Similarity matrix was subjected to analysis using NTSYS-pc software (Rohlf, 1998). The corresponding dendrogram was created by applying unweighed pair-group method with arithmetical averages (UPGMA) cluster analysis and cophenetic correlation was computed to check the goodness of fit for clusters (Rohlf, 1998). Number of polymorphic bands, percentage of polymorphism and polymorphic information content (PIC) in each species were calculated for each primer. PIC; the probability of detection of polymorphism by a primer between two randomly drawn genotypes was calculated according to formula:

$$PIC = 1 - \sum {p_i}^2$$

where, p_i is the frequency of i^{th} allele (Sehgal *et al.*, 2009b). The maximum values of PIC for dominant and co-dominant markers are 0.5 and 1.0, respectively (Sehgal *et al.*, 2009b). Nei's gene diversity, Shannon's index, number of polymorphic loci and percentage of polymorphic loci for among accessions was calculated using population genetic analysis software (POPGENE.32) (Yeh *et al.*, 1997).

RESULTS

Table 2

Name, sequences, total number of produced band, number of polymorphic band and PIC for the ten used ISSR primers in the genus *Carthamus*

Primer	Sequences (5'……..3')	Total number of produced bands	Number of polymorphic bands	PIC
ISSR1	$(GA)_7$-RG	13	13	0.295
ISSR2	$(CA)_7$-YC	15	15	0.254
ISSR3	$(AG)_8$-T	23	23	0.288
ISSR4	$(AG)_8$-YC	12	12	0.317
ISSR5	$(GT)_8$-YC	24	24	0.341
ISSR6	$(AC)_8$-YG	13	13	0.313
ISSR7	$(TG)_8$-RC	20	20	0.294
ISSR8	$(AT)_7$-RC	-	-	-
ISSR9	$(CA)_7$-YG	10	10	0.367
ISSR10	$(CA)_8$-RC	14	14	0.273
Total		144	144	
Average		16	16	0.305

Out of ten 10 used ISSR primers, 9 showed polymorphism and produced a total of 144 polymorphic bands (Table 2). The number of polymorphic products ranged from 10 in ISSR9 to 24 in ISSR5 that implied ISSR markers could detect variation at DNA level for these plant materials. PIC, polymorphism information content, ranged from 0.254 to 0.367, which the least and most informative primers were ISSR2 and ISSR9, respectively (Table 2). To compare relative ability of primers in detecting polymorphisms among the samples of each species, PIC of each ISSR primer for all genotypes were calculated separately (Table 3). PIC was not identical for

all species, thus some primers were more efficient in detecting polymorphism for one species when compared to the others (Table 3). Except primer ISSR2, all primers were considerably able to detect polymorphism in *C. tinctorius*, cultivated safflower (Table 3). The best primers for detecting polymorphism in population of *C. lanatus* were ISSR9 and ISSR10, and the best for population of *C. oxyacanthus* were ISSR2, ISSR3 and ISSR7 (Table 3).

Table 3

ISSR primers, number of total produced band, number of polymorphic band and polymorphism information content (PIC) for each genotype. a, b, c and d are cultivated genotypes, *C. oxyacanthus*, Isfahan and Tehran ecotypes of *C. lanatus,* respectively

Genotype	ISSR1				ISSR2				ISSR3			
	a	b	c	d	a	b	C	d	a	b	C	D
Number of produced band	9	2	6	9	3	5	12	4	11	13	15	7
Number of polymorphic bands	6	0	6	8	1	1	12	4	10	13	15	7
PIC	0.26	0	0.42	0.3	0.07	0.09	0.32	0.38	0.32	0.37	0.34	0.37
Number of produced bands	5	3	10	11	7	14	14	11	7	5	9	3
Number of polymorphic bands	4	1	10	10	3	7	14	9	5	1	9	2
PIC	0.26	0.05	0.36	0.27	0.19	0.09	0.3	0.3	0.25	0.09	0.31	0.25
Number of produced bands	7	7	9	15	5	7	7	5	3	5	6	9
Number of polymorphic bands	6	7	9	14	3	7	7	5	2	5	6	9
PIC	0.36	0.37	0.38	0.36	0.23	0.4	0.25	0.39	0.28	0.46	0.29	0.31

To estimate genetic similarity coefficient (GS) between the genotypes of different *Carthamus* species, all 144 polymorphic bands were used in the analysis. The GS values ranged from 0.222 to 0.946 for all the samples (Fig. 1). The highest GS were observed between genotypes Aceteria and 541-5 from *C. tinctorius* and also between two samples of *C. lanatus* (Fig. 1).

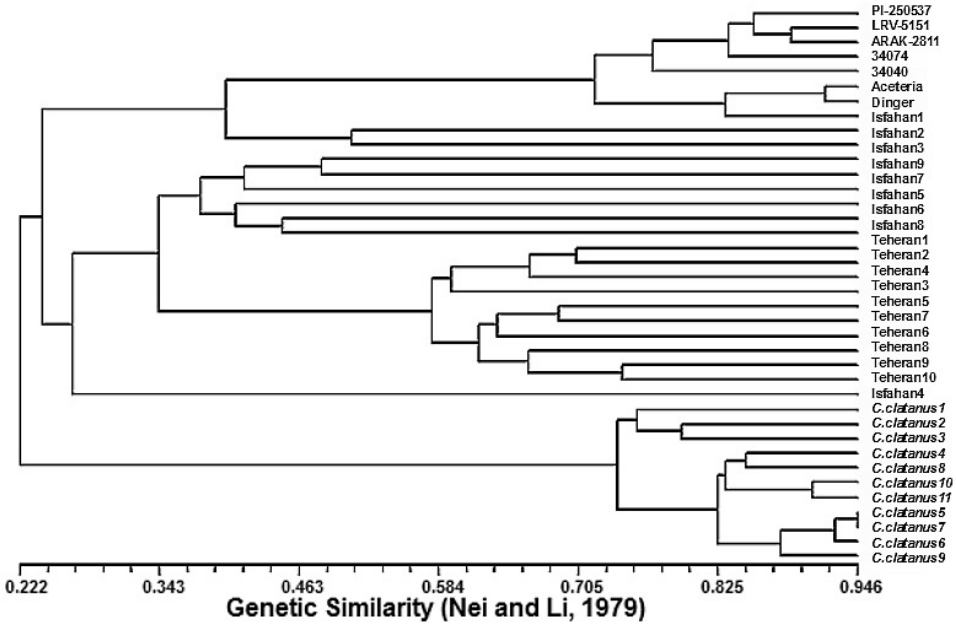

Fig.1. UPGMA-based dendrogram showing genetic similarity between 38 samples of three species of the genus *Carthamus* based on GS values

Table 4

Nei's gene diversity, Shannon's index, number of polymorphic loci and percentage of polymorphic loci for three species of the genus *Carthamus*

Accession	Nei's gene diversity		Shannon's index		Number of polymorphic loci	Polymorphic loci [%]
	Mean	SD	Mean	SD		
Cultivated safflower	0.10	0.18	0.15	0.26	40	70.17
C. lanats	0.09	0.17	0.14	0.25	39	63.93
C.oxyacanthus (Isfahan`s ecotype)	0.15	0.16	0.25	0.23	88	100,00
C.oxyacanthus (Tehran`s ecotype)	0.15	0.19	0.22	0.27	68	91.89

To understand the amount of genetic similarity among these three species, Nei's gene diversity, Shannon's index, number of polymorphic loci and percentage of polymorphic loci was calculated (Table 4). Nei's gene diversity varied from 0.09 to 0.15 and the highest belonged to *C. oxyacanthus* populations (Table 4). Shannon's index, ranged from 0.14 (*C. lanatus*) to 0.25 (*C. oxyacanthus*, Isfahan), showed that samples of Isfahan ecotype had more variation than others. Percent of polymorphic loci was from 63.93 for *C. lanatus* samples to

100% for C. *oxyacanthus* (Isfahan). All obtained association parameters showed that Isfahan ecotype of C. *oxyacanthus* had the highest variation at DNA level.

The results of UPGMA cluster analysis for data produced by ISSRs presented as dendrogram (Fig. 1). Based on ISSRs data, the dendrogram divided the 38 samples of *Carthamus* into three major clusters (Fig. 1). Populations of *C. tinctorius* and *C. oxyacanthus* had more genetic similarity and grouped together in one cluster. The highest distance was observed between *C. tinctorius* and *C. lanatus* populations. The first cluster contains all cultivated genotypes along with two samples of *C. oxyacanthus* (ecotype Isfahan). The second cluster corresponds to all individuals sampled from *C. oxyacanthus* (ecotypes Tehran and Isfahan). The last distance-based cluster includes individuals of *C. lanatus*.

DISCUSSION

The results indicate that ISSR markers could effectively detect diversity and genetic relationships among wild and cultivated species of the genus *Carthamus*. Except for some samples of wild populations, clustering the genotypes based upon ISSR produced data matched the species boundaries in the genus *Carthamus*. Increased genus sampling and analysis of ISSR data have led to a better understanding of relationships within *Carthamus*. Sabzalian *et al.* (2009) also classified the Iranian populations of *Carthamus* based on ISSR markers in addition to agro-morphological traits. Also, our results showed a considerable intra and inter variation among *C. oxyacanthus* for most used ISSR markers. This high diversity may be due to several items such as the pollination system, insect activities and gene flow with cultivated safflower. Knowles (1980) suggested *C. oxyacanthus* is mixed of self-compatible and self-incompatible individuals and therefore the amount of heterogeneity in the population is high. In other hand this wild species is crossable with domesticated safflower and gene flow between these accessions by insect activities especially in Iran, where they are sympatric, is not impossible (Ashri and Knowles, 1960; Mayerhofer *et al.*, 2011). Previous studies also hypothesized that *C. oxyacanthus* is the progenitor to the cultivated *C. tinctorius* (Chapman and Burke, 2007). Knowles (1976) deduced that the distribution of both species in the Near East is consistent with safflower originating in this region. However, *C. oxyacanthus* is morphologically distinct and in addition to some differences in seed morphology, this species has more spines and smaller flower heads than *C. tinctorius* (Bagmohammadi, 2011). Our data also supported this reality, where two samples of *C. oxyacanthus* (ecotype Isfahan) grouped with cultivated genotypes in the same cluster. Our results imply that this species could introduce favorable genes to cultivated safflower via inter-specific hybridization in breeding programs.

For *C. oxyacanthus*, variation among samples of Tehran`s ecotype was lower than Isfahan`s ecotype. This difference may be due to their harvesting place, as seeds of Tehran`s ecotype were collected from a mountain area where gene flow is more limited. MacPherson *et al.*, (2004) suggested that for preventing gene flow between transgenic safflower genotype with wild weedy relatives, locations where wild species of *Carthamus* have not been naturalized may provide biologically isolated area.

In this study, all samples of *C. lanatus* were clustered together in one group. The Shannon index also indicated that there was a large variation in this population (0.14), but these differences seem not to be enough. The results presented here may be used in such studies in the future to target diverse groups in the *C. lanatus* population. Cultivated genotypes and *C. oxyacanthus* populations clustered differently from *C. lanatus*, so gave some indication of the variability between these related species. As mentioned by Heaton and Klisiewicz (1981), obtaining hybrid plants followed by treating the rescued embryos with colchicine to double the chromosome number and producing a fertile autopolyploid (n = 34) is prerequisite for successful hybridization between *C. lanatus* and cultivated safflower.

Safflower molecular breeding is lagging behind other oilseed crops. The creation of a safflower saturated genetic map is an essential requisite for molecular breeding programs. The ISSR resources developed in this research along with those recently developed (Chapman *et al.*, 2009; Naresh *et al.,* 2009; Mayerhofer *et al.*, 2010) will contribute to construct a safflower genetic map with a density sufficient for basic and applied research and to advance in safflower molecular breeding. In conclusion, ISSRs can be used to establish foundation for molecular marker assisted breeding of *Carthamus* resource based on our analysis. The genetic diversity obtained from the study can also be applied to the selection of parents for generating mapping populations and to identify intra and inter-specific differences.

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