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Full length Research paper

Investigation of Genetic Diversity in Ethiopian Collections of Safflower (*Carthamus tinctorius*) as revealed by ISSR markers

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Safflower (*Carthamus tinctorious* L.) is an oilseed crop that is valued as a source of high quality vegetable oil. Among the genus *Carthamus*, safflower is the only cultivated species. The objective of this study was to investigate the genetic diversity of safflower accessions from Ethiopia using ISSR molecular markers. In this study, the genetic diversity of 70 safflower genotypes originated from different geographical regions of Ethiopia was evaluated using four ISSR primers. DNA was extracted from selected plants per accession using a triple CTAB extraction technique. The four selected ISSR primers produced a total of 43 bands of which 87.5% were polymorphic. Among regions, safflower population from Oromia showed 0.32 and 0.48 gene diversity and Shannon diversity index, respectively, with the highest percentage of polymorphism (86.1%).The highest gene diversity (0.37) and Shannon index (0.55) was shown by the only tetra-nucleotide [$(GACA)_4$, primer 873]. Moreover, AMOVA showed that 98.9% of the variation is attributed to within population while only 1.1% is among populations variation. The results reveal the presence of higher genetic diversity that deserves conservation attention and sustainable use strategy to improve the productivity of safflower in Ethiopia.

Key words: *Carthamus tinctorius* L, genetic diversity, ISSR molecular marker, polymorphism, Ethiopia.

INTRODUCTION

Safflower, *Carthamus tinctorius*, L. is an oilseed crop that belongs to the family Asteraceae. The genus *Carthamus* has 25 species, of which *C. tinctorius* is the only cultivated one with 2n = 24 chromosomes (Helm *et al.,* 1991). It is dicotyledonous, herbaceous, and annual plant. It has colorful flower heads, a deep taproot, and the production of white, oil-bearing seeds. It has adapted to grow in hot, dry climates, and well-drained soil (Deokar and Patil, 1980). Safflower is believed to have been domesticated somewhere in the Fertile Crescent region over 4000 years ago (Ashri, 1975; Knowles, 1969). However, Vavilov (1951) considered Ethiopia, Afghanistan and India as the primary centers of origin of safflower. Currently, it is grown as an oilseed crop in over 60 countries worldwide and India as the largest producer of safflower (Weiss, 2000).

Safflower is mainly cultivated for its seed, which is used primarily for edible oil. In the past, the crop is grown for its flowers used for coloring and flavoring foods, making dyes and medicine. In Ethiopia, boiled and finely pounded safflower kernels are mixed with water and the supernatants is used to prepare the so called '*fitfit*', which is used as fasting-food. Roasted seeds mixed with roasted chickpeas, barley or wheat, are eaten as a snack food in Ethiopia and Sudan (Dajue and Mundel, 1996). Ethiopian safflower is very much neglected and it is cultivated only as a minor oil crop in marginal land with very limited information available on its genetic resources. In Ethiopia, safflower cultivation is mostly done by small holder farmers around homesteads (Edwards and Sue, 1991).

DNA markers become the marker of choice for the study of crop genetic diversity (Karp, 2002). Genetic

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Table 1. List of primers, sequence, annealing temperature and amplification pattern.

Primers	Annealing T°	Sequence	Amplification pattern
810	45	5’-GAGAGAGAGAGAGAGAT-3’	Reproducible but not polymorphic
812	45	5’-GAGAGAGAGAGAGAGAA-3’	Good
818	48	5’-CACACACACACACACAG-3’	Good
824	48	5’-TCTCTCTCTCTCTCTCG-3’	Polymorphic but not reproducible
834	45	5’-AGAGAGAGAGAGAGAGYT-3’	Reproducible but not polymorphic
844	48	5’-CTCTCTCTCTCTCTCTRC-3’	Polymorphic but not reproducible
872*	38	5’-GATAGATAGATAGATA-3’	Polymorphic but not reproducible
873*	45	5’-GACAGACAGACAGACA-3’	Good
874*	48	5’-CCCTCCCTCCCTCCCT-3’	Polymorphic but not reproducible
880**	45	5’-GGAGAGGAGAGGAGA-3’	Good

Source: Primer kit 900 (UBC 900); Single-letter abbreviations for mixed base positions: R= (A, G) Y = (C, T), *Tetra-nucleotide primers, **Penta-nucleotide primer and the rest are di-nucleotides.

diversity of some safflower germplasm has been previously investigated based on agro-morphological markers were the first to be used for genetic diversity study (Jaradat and Shahi, 2006).

RAPD markers were also applied to study the genetic diversity of *C.tinctirius* landraces germplasm collected from Iran (Mahasi *et al*., 2009) while ISSR marker has been used by Ash *et al.* (2003) and Yang *et al.* (2007) to investigate relationship among *C.tinctirius* germplasm collected from different part of the world. None of the above studies either focused on Ethiopian germplasm or include samples from Ethiopia. Therefore, this study aimed at providing valuable information on the levels of divergence within and among populations of *C. tinctorius* from Ethiopia and generate information to design conservation strategy for this neglected but important crop.

MATERIALS AND METHODS

Plant Materials and Genomic DNA Extraction

Seeds of 70 safflower accessions collected from four different administrative regions of Ethiopia were obtained from Ethiopian Institute of Biodiversity (EIB), Addis Ababa, Ethiopia.

All the 70 safflower accessions seeds samples were grown in a greenhouse and fresh leaves from five week old plants were used for genomic DNA extraction. Five young leaves were collected separately from five randomly selected individual plants of each accession just before flowering and equal proportions of leaves were grinded for genomic DNA extractions. Genomic DNA was extracted from leaf samples following Borsch *et al*. (2003).

Primer Selection and Optimization

A total of 10 ISSR primers that were available at Genetics Laboratory were used for the initial testing of variability and reproducibility. For optimization and screening of primers, one individual was selected from each population with 1:5 dilutions and concentration was adjusted after running test gel using 0.89% agaros. All pre-selected 10 primers were used for reproducibility and polymorphism test and evaluation. Finally, two di-nucleotide primers (primer 812 and 818), one tetra-nucleotide primer (primer 873) and one penta-nucleotide primer (primer 880) (Table 1) were selected based on polymorphism and reproducibility.

DNA Amplification

ISSR-PCR amplification based on four primers were carried out following the reaction mixture below. PCR amplification was carried out in a 25 µl reaction mixture containing1µl template DNA, 13.45µl ddH20, 5.6µl dNTP (1.25mM), 2.6µl Taq buffer(10xThermopol reaction buffer), 1.25µl Mgcl2 (50mM), 0.6µl primer (20pmol/µl) and 0.5µl Taq Polymerase (5u/µl).

The amplification program was performed in a Biometra version 3.10 TPersonal using 48 well plates using 2 minutes preheating and initial denaturation at 94^{0}C, followed by a regular cycling event of 40 cycles of 20 seconds denaturation at 94°C, 1 minute primer annealing at (45^{0}C/ 48^{0}C) based on primers used,1.30 minutes extension at 72^{0}C and final extension for 7 minutes at 72^{0}C. The PCR reactions were also stored at 4^{0}C until loading on gel for electrophoresis.

Table 2. Fingerprint patterns generated using four ISSR primers selected for this study.

Selected Primer	Repeated motif	No. of clear scorable bands	Amplification Pattern	ISSR gel observation
812	(GA)8A	12	Good	Good
818	(CA)8G	6	Good	Good
873	(GACA)4	15	Best	Good
880	(GGAGA)3	10	Good	Good
		43		

Source: primer kit 900 (UBC 900); Single-letter abbreviations for mixed base positions.

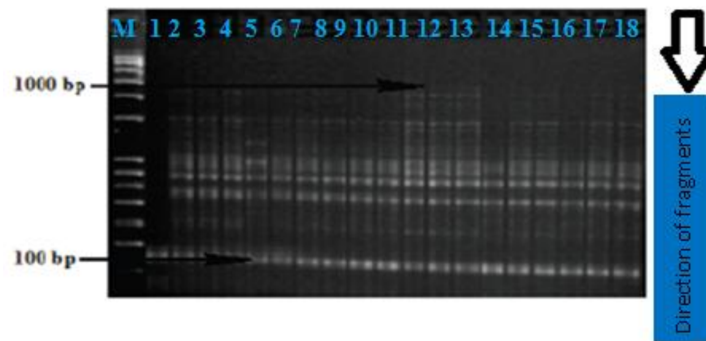

Figure 1. ISSR amplification profile for primer 873 on *Carthamus tinctorius* accessions. Numbers represent the accessions *from Amhara region.* **M:** 100bp DNA ladder.

Table 3. Number of polymorphic loci, percent polymorphism, Nei's gen diversity and Shannon index of safflower with all primers.

Population	No. of accessions	With all primers			
		NPL	PP (%)	Gene diversity (h)	Shannon index(I)
Amhara	33	29	67.4	0.27±0.12	0.39±0.29
Oromia	28	37	86.1	0.32±0.15	0.48±0.21
SNNPRs	4	9	20.9	0.08±0.16	0.11±0.23
Tigray	5	15	34.9	0.16±0.12	0.22±0.31
Overall	70	90	52.3	0.21±0.18	0.30±0.26

ISSR Gel Electrophoresis and Visualization

The quantity and quality of genomic DNA was tested using 0.89% agarose gel electrophoresis. Stock solution of 10X Tris Borate EDTA (TBE), commonly used electrophoresis buffer: 108 gm Trisbase; 55 gm Boric acid; 40ml EDTA, pH 8.57 components per liter was prepared and stored at room temperature. From the stock, working solution of 10X TBE prepared and then further diluted to 1x TBE and used to prepare the gel as well as fill the electrophoresis tank.

The ISSR gel with 1.67% was prepared by boiling 1xTBE in 500 ml Erlenmeyer flask in microoven for 2:30 minutes. After the agarose solution cooled down at room temperature, 12µl of Ethidium bromide was added for better visualization of the gel using UV light. Then it was poured on to the gel tray and the comb was inserted immediately after the agarose was poured and the gel was left for three hours to set and solidified properly. The comb was carefully removed and put the gel tray in to the electrophoresis tank properly filled with electrophoresis buffer, 1XTBE and then the amplified ISSR product of 8µl was loaded onto ISSR gel with 2µl loading dye. DNA ladder 100 bp was also used to estimate molecular weight. The electrode was connected and the power supply was turned on; the voltage was adjusted at 100 V and left for three hours. The electrophoresis gel was photographed using UV dual-intensity transluminator, Zenith, Canon camera connected with computer. But gel

Table 4. Number of polymorphic loci, percent polymorphism, Nei's genetic diversity and Shannon index of safflower with each primer.

Individual primers				
Primers	NPL	PP (%)	Gene diversity (h)	Shannon index (I)
812	8	66.7	0.24±0.20	0.36±0.28
818	6	100	0.23±0.05	0.39±0.06
873	14	93.3	0.37±0.12	0.55±0.17
880	9	90	0.36±0.17	0.52±0.22
Overall	37	87.5	0.30±0.13	0.46±0.18

NPL= number of polymorphic loci, PP (%) = percent polymorphism, h= Nei's gene Diversity and I= Shannon index information for each population and over all populations.

picture was taken after staining with 25µl Ethidium bromide (10mg/ml) which was mixed with 250 ml double distilled water for 30 minutes and washed for 30 minutes using double distilled water. Different photographs using different lens aperture, were taken and saved for later scoring.

Data Scoring and Statistical Analysis of Diversity

Although a large number of fragments were generated from each primer, only clearly distinguishable and reproducible bands were selected and data were entered in a computer file as a binary matrix "0" coded for absence and "1" for presence of a band. Data from the ISSR studies was analyzed using various statistical programs. POPGENE version1.32 software (Ye,1999) was used to calculate genetic diversity for each population as number of polymorphic loci, percent polymorphism, mean of Nei's genetic diversity and Shannon index. Analysis of molecular variance (AMOVA) was used to calculate variation among and within population using Areliquin version 3.01 (Excoffier *et al*., 1992).

RESULT AND DISCUSSION

ISSR Polymorphism

Out of the 10 primers tested initially, four of them with clear and reproducible banding pattern were selected and used in this investigation (Table 2). The molecular weight of the bands amplified using the four primers were in the range of 100 to 1000bp (Figure 1). Four ISSR primers produced fragment that ranged from 6 to 14 which were total generated 43 bands (Table 2) across the 70 accessions, of which 37 were polymorphic (Table 4). The polymorphic bands of the primers ranged from six (primer 818) to 14 (primer 873) across the genotypes. The average polymorphic bands per primer were 10.75. The percentage of polymorphism for primers ranged from 66.7 to 100, with an average polymorphism percent of 87.5% (Table 4). In line with the results of the present study, similar result was obtained by Seyed *et al.* (2010), whereby higher percent of polymorphism (96%) was revealed with ISSR marker, which was higher than that of RAPD (81.08%). In the present study, among all populations studied, the Oromia population were found to have higher percentage polymorphism, 86.05% and followed by population from Amhara, 67.44%. In this investigation, the di-nucleotide primers, namely 812 and 818 were observed to have eight and six polymorphic loci, respectively. The tetra-nucleotide [$(GACA)_4$, primer 873] and penta-nucleotides [$(GGAGA)_3$, primer 880] were observed to have 14 and 9 polymorphic loci, respectively. Similar to our results, Yang *et al.* (2007) has reported that ISSR primers produced the highest polymorphic bands in safflower.

Our data indicated that ISSR technology can detect considerable polymorphisms (87.5%) in our genotypes, suggesting that it will be useful in safflower germplasm characterization and fingerprinting purposes. This study provides fundamental evidence that ISSR marker is a simple, informative, reproducible and suitable approach to evaluation of molecular diversity in safflower.

Population Genetic Diversity

Various measures of genetic variation are presented in the Table 3 and 4. Nei's gene diversity (H) values showed overall 0.30±0.13. Similarly, the Shannon's information indices (I) were 0.46±0.18 (Table 4). Among cultivated safflower populations considered in the present study, Oromia showed higher gene diversity (h=0.32) and Shannon index (I=0.48) as compared to the other three populations of cultivated safflower. Safflower populations from Amhara regions showed 0.27 and 0.39 values of gene diversity and Shannon index, respectively. In terms of primers, the highest gene diversity (0.37) and Shannon index (0.55) was shown by The tetra-nucleotide [$(GACA)_4$, primer 873] and followed by penta-nucleotides [$(GGAGA)_3$, primer 880] with gene diversity and Shannon index value of 0.36, 0.52, respectively (Table 4) and the two motif primers of $(GACA)_4$ and $(GGAGA)_3$ were produced the highest mean of polymorphism. Both diversity indexes, gene diversity measurements and Shannon's indices, showed high polymorphism within and among Ethiopian safflowers.

Table 5. Analysis of Molecular Variance (AMOVA) of safflower populations in Ethiopia.

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation index	P
Among populations	3	14.235	0.04492Va	1.1	0.01	0.0
Within populations	66	271.022	4.10639Vb	98.9		0.0
Total	69	285.257	4.15131			

Analysis of Molecular Variance (AMOV) and Genetic Partitioning

When data are available from more than one population, it is usually of interest to evaluate the degree to which the total gene diversity partitions into within and between population components (Lynch and Milligan, 1994). In this study, analysis of molecular variance revealed that higher percentage of variation is attributed to variation within populations (98.9%) and only 1.1% of the variation was attributed to difference among populations (Table 5).

An understanding of the partitioning of genetic variation within crop gene pools can provide insight into the evolution of crop lineages. In addition to its implication for conservation, partitioning the genetic variation in to its components has significant impact in the future breeding and conservation plan. In this investigation, the AMOVA analysis revealed a significant difference between and within four groups of safflower genotypes. Yang *et al.* (2007) has reported that AMOVA revealed a non-significant difference between two groups of genotypes (native *vs.* exotic).

The one reason for high significant genetic variation within safflower population in the present study, could be due to floral biology and pollination nature of safflower. Moreover, insect pollinators including bees and birds might be attracted due to its colorful flower that facilitate gene flow among populations. Not only the pollination nature of safflower, but also the existence of seed exchange among local farmers could also be the additional factor. Moreover, the presence of long distance marketing of safflower within and among different regions by farmers and local traders have their own effect on the observed variation of cultivated of safflower. Hamrick and Godt (1989), stated that, the biological characteristics of a species, population structure, mating system and multiple evolutionary process (genetic drift: bottleneck and founder effect) are determine the level and pattern of genetic variation observed in natural population.

CONCLUSION

It is evident from this study that the ISSR assay can be useful for safflower germplasm characterization and evaluation of its genetic diversity. The study also clearly revealed the existence of higher genetic diversity within Ethiopia safflower and pointed towards populations that deserve conservation attention. Moreover, additional collection mission is required in the future to assemble more population for conservation and use.

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