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EVALUATION OF GENETIC DIVERSITY IN GERBERA GENOTYPES REVEALED USING SCOT AND CDDP MARKERS

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Abstract

Genetic diversity in the germplasm of flowers is a key to genetic improvement of ornamental species. Gerbera cultivars are grown commercially worldwide and their flowers are durable and appealing, having a wide variety of colors. Genetic diversity based on molecular analysis can provide useful information for germplasm management and varietal characterization. In this study, we used start codon targeted (SCoT) and conserved DNA-derived polymorphism (CDDP) to assess the genetic diversity among 22 gerbera cultivars. Our findings showed that average polymorphism information content (PIC) was 0.39 and 0.40 for SCoT and CDDP markers, respectively, indicating that the studied markers were equal in terms of assessing genetic diversity. The results of clustering for both marker systems grouped the genotypes into three clusters. We found a positive significant correlation ($r = 0.73$, $P < 0.01$) between similarity matrix gained by both SCoT and CDDP markers. Cluster analysis for the CDDP and SCoT markers grouped the cultivars in three clusters. Average Marker index (MI) for SCoT and CDDP was calculated 3.40 and 2.45, respectively. This is the first time that the efficiency of SCoT and CDDP markers, as a novel method, have been compared with each other to evaluate genetic diversity in a set of gerbera genotypes. Our results showed that the comparison of different genetic diversity estimation methods could be useful for the improvement and crossing of gerbera genetic resources. This information can be used for the selection of superior genotypes for gerbera breeding programs.

Abbreviations: SCoT – start codon targeted; CDDP – conserved DNA – derived polymorphism; MI – marker index.

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Introduction

Gerbera jamesonii, belonging to *Asteraceae* family, is the fifth most used cut flower in the world originating from South America, Africa, and Asia (NESOM 2004). This plant is perennial and reproduces asexually. Gerbera was domesticated as a result of a cross between *Gerbera jamesonii* and other African *Gerbera viridifolia* species (HANSEN 1985). Gerbera is one of the most popular and beautiful flowers used as a decorative garden plant or as cut flowers and varies in shape, size, and color (white, yellow, orange, red, and pink). Determination of available genetic diversity for the characters of economic importance is very useful in crop breeding. Traditionally, breeding of gerbera is based on hybridization among cultivars and phenotypic selection of the best progenies, followed by clonal dissemination of released cultivars (PRAJAPATI et al. 2014, DE PINHO BENEMANN et al. 2013). However, selection is effective only when the observed traits in population is heritable in nature. Thus, achievement of proper genotype selection depends on important parameters such as variation, estimates of heritability, and genetic progress in gerbera germplasm. Genetic diversity based on molecular analysis can provide useful information for germplasm management and varietal characterization. Researchers have used different tools to evaluate genetic variations such as morphological, isozyme marker, and molecular markers. Morphological analysis is the first step in the classification and description of any plant germplasm (UPADHYAYA et al. 2001, GHAFOOR et al. 2001) which is one of the easiest methods for classical plant breeders in selecting desirable traits. Morphological evaluations are inexpensive and easy but are extremely influenced by environmental conditions. Molecular markers are useful tools to assess genetic diversity and provide an efficient mean to link phenotype and genotype variations (SOUFRAMANIEN and GOPALAKRISHNA 2004, VARSHENY et al. 2005). On the other hand, molecular markers are appropriated tool for measuring diversity of plant species which makes it possible to precisely characterize genotypes and provide measurement of genetic relationships (GHAFOOR et al. 2001). Genetic diversity in gerbera has been analyzed using a wide range of molecular marker systems such as EST-SSR (GONG and DENG 2010), RAPD (PRAJAPATI et al. 2014), and ISSR (LI et al. 2004) and AFLP. However, many of these markers used for genetic diversity and population structure are considered to be not so much efficient. For example, disadvantages of RAPD include being dominant, non-reproducible, and lack of detection of allelic system (MIAH et al. 2013). Although, SSR markers have been utilized to develop diversity fingerprinting in gerbera however, development of SSR markers require sequence information

and may not be suitable across species (CHEN et al. 2016). Thus, new markers such as CDDP and SCoT have been developed which can be considered as proper alternatives for previous markers (GUPTA and RUSTGI 2004).

SCoT markers are one of the reliable techniques and have several advantages over other markers such as higher efficiency, more informative, and even inexpensive. Primers used in this method are designed according to short conserved region surrounding the ATG translation start (or initiation) codon, showing the correlation between functional genes and their corresponding traits. Hence, this method has been successfully applied in different plant species to explore their genetic variability (AGRAWAL et al. 2019, ZHANG et al. 2015). SCoT and CDDP markers are functional markers (FM) which are usually dominant and reproducible (POCZAI et al. 2013). Although SCoT and CDDP are dominant markers, however, a number of co-dominant markers are also generated during amplification which can be used for genetic diversity analysis. SCoTs can be used for assessing genetic diversity and to obtain reliable information about population processes and structure across different plant families (COLLARD and MACKILL 2009a). CDDP and SCoT markers can yield many detectable polymorphic bands. The techniques are based on single primer with a high annealing temperature which leads to improved reproducibility.

CDDP (COLLARD and MACKILL 2009a) and SCoT (COLLARD and MACKILL 2009b) markers were developed based on the conserved regions of genes which have typically functional domains corresponding to conserved DNA sequences within gene regions (POCZAI et al. 2013). SCoT and CDDP have longer primers with higher annealing temperature requirement which will make them more reliable and reproducible than the arbitrary markers such as RAPD. CDDP and SCoT markers have been used to investigate genetic diversity in wide range of plant species (HAMIDI et al. 2014, HAJIBARAT et al. 2015, SAIDI et al. 2017). The use of CDDP and SCoT markers for studying genetic diversity are reported here for the first time for gerbera genotypes. The aims of the present study were to determine the efficiency of CDDP and SCoT markers and the comparison of these markers for estimating genetic diversity and relationships of gerbera genotypes.

Material and Methods

Plant material and genomic DNA extraction

A total of 22 Dutch gerbera cultivars obtained from the National Institute of Ornamental Plants (NIOP), Mahallat, Iran were surveyed in this study (Table 1). Genomic DNA was extracted from 1 g of leaves of each

Table 1

Names of the studied cultivars in this research

No	Genotype	Flower	No	Genotype	Flower
1	Rosalin		11	Duble Dutch	
2	Sorbet		12	Cacharlle	
3	Souvenir		13	Hooper	
4	Dune		14	Nuance	
5	Intense		15	Quote	
6	Aqua melone		16	Esmara	
7	Edelweiss		17	Sazo	–
8	Carambole		18	Pink elegance	
9	Balance		19	Essendre	
10	Stanza		20	Cabana	
21	Klimanjaro		22	Red-417	

cultivar using the DNA isolation method for gerbera based on the modified CTAB method (Lassner et al. 1989). Leaf samples were crushed using 5 ml of extraction buffer (EDTA, 1 M TrisHCl pH 8.0, 3 M NaCl) and, 1% CTAB, 0.7 M NaCl and 5 ml H_2O) and incubated for 1 h at 65°C. The extracted solution was treated with equal volume of Chloroform: Isoproponol mixture (1:1; v/v). DNA pellet was then treated with double volume of ice cold Isopropanol and washed twice with 76% ethanol. The isolated DNA was air dried and stored at −20°C in ddH_2O.

SCoT marker analysis

SCoT markers were amplified through PCR by nine SCoT primers as listed in Table 2. Thermal cycling included 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min annealing at 48°C, 2 min at 72°C and ending by an extension for 10 min at 72°C. The PCR products were separated on 1.5% agarose gel and stained with ethidium bromide. The polymorphic primers were then used for further analysis of 22 gerbera cultivars (Table 2).

Table 2

Primers used in SCoT and CDDP marker systems for study of genetic variation among 22 gerbera cultivars

Type	Primer	sequence 3′ → 5′	% GC	Tm
SCoT	SCoT1	CAACAATGGCTACCACCA	50	50
	SCoT2	CAACAATGGCTACCACCC	55	50
	SCoT13	ACGACATGGCGACCATCG	61	50
	SCoT22	AACCATGGCTACCACCAC	55	50
	SCoT28	CCATGGCTACCACCGCCA	66	50
	SCoT35	CATGGCTACCACCGGCCC	72	50
	SCoT36	GCAACAATGGCTACCACC	55	50
	SCoT 13	ACGGACATGGCGACCATCG	61	50
	SCoT 20	ACCATGGCTACCACCGCG	66	50
CDDP	KNOX-02	CACTGGTGGGAGCTSCAC	67	59
	KNOX-03	AAGCGSCACTGGAAGCC	68	58
	MYB-02	GGCAAGGGCTGCCGG	80	54
	WRKY-R1	GTGGTTGTGCTTGCC	60	51
	WRKY-R2	GCCCTCGTASGTSGT	64	52
	WRKY-R3	GCASGTGTGCTCGCC	65	53
	ERF1	CACTACCCCGGSCTSCG	77	56
	ERF2	GCSGAGATCCGSGACC	77	57
	HEP-VQ	CACGAGGACCTSCAGG	69	51

PCR amplification was performed in 20-µL reactions containing 30 ng of template DNA, 1 × PCR buffer, 0.2 mmol L^{-1} dNTPS, 0.4 µmol L^{-1} of primer, and 500 U of Taq polymerase (Cinaclon, Iran). The PCR reaction was performed in a PCR thermocycler (Master Cycler Gradient, Eppendorf) as follows: 95°C for 4 min, followed by 38 cycles of denaturation at 94°C for 45 s, annealing at 49°C for 45 s, and extension at 72°C for 2 min. A final extension cycle at 72°C for 10 min followed. The PCR products were separated in 1.3% agarose gels and stained with ethidium bromide.

CDDP marker analysis

CDDP marker was amplified through PCR using nine CDDP primers as listed in Table 2. PCR amplification was performed in 20-µL reactions containing 30 ng of template DNA, 1 × PCR buffer, 0.25 mmol L^{-1} dNTPS, 0.35 µmol L^{-1} of primer, and 500 U of Taq polymerase (Cinaclon, Iran). The PCR reaction was performed in a PCR (Master Cycler Gradient, Eppendorf) as follows: 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 49°C for 45 s and extension at 72°C for 2 min. A final extension cycle at 72°C for 10 min followed. The PCR products were separated in 1.5% agarose gels and stained with ethidium bromide. These primers (up to 18-mer) and their GC content ranged between 61 and 74%. CDDP primers were selected for final amplification based on GC content of 50–60% and an annealing temperature of 48°C (Table 2). All amplified products were resolved on 1.3% agarose gel made in 1x TBE buffer. The electrophoresis was performed for 45 min at 95 V and visualized with ethidium bromide. The image of banding patterns was captured under UV light using gel documentation system.

Data analysis

The amplified SCoT and CDDP markers were scored for presence (1) or absence (0) of bands. Only clear and reproducible bands were scored. Marker index (MI) was calculated as given by VARSHNEY et al. 2005:

$$MI = PIC \cdot Poymorphic\ band$$

PIC value for each polymorphic locus was estimated according to Roldan-Ruzi (ROLDÁN-RUIZ et al. 2000). PIC = 1 – S(Pij)2; where Pij is the frequency of the ith pattern showed by the jth primer aggregated across all patterns revealed by the primers (BOTSTEIN et al. 1980). NTSYS was applied for analyzing pairwise genetic distances and for making the distance matrix (ROHLF 1998). Genetic similarity among samples was eva-

luated by calculating the Jacard similarity coefficient and dendrogram analysis using the un-weighted neighbor-joining method (UNJ). Mantel's test (MANTEL 1967) for Jaccard coefficients was performed to compare each pair of similarity matrices created by NTSYSpc version 2.0.

Results

SCoT analysis

The nine SCoT primers produced a total of 53 reliable fragments of which 48 bands were polymorphic. Each primer produced an average of eight bands. SCoT1 yielded the maximum number of polymorphic bands and SCoT20 produced the minimum number of fragments (Table 3).

Table 3

Polymorphism detected with 18 CDDP and SCoT primers in fifteen collections of gerbera cultivars

NO	Primer	PB	MB	TB	PIC	MI	PPB%
CDDP	KNOX-02	8	1	9	0.43	3.48	88
	KNOX-03	5	3	8	0.43	2.16	62
	MYB-02	10	0	10	0.44	4.43	100
	WRKY-R1	10	0	10	0.43	4.33	100
	WRKY-R2	4	3	7	0.41	1.64	57
	WRKY-R3	3	0	3	0.28	0.85	100
	ERF1	10	0	10	0.43	4.39	100
	ERF2	6	0	6	0.39	2.35	100
	HEP-VQ	7	0	7	0.41	2.91	100
SCoT	SCoT 1	14	0	14	0.45	6.4	100
	SCoT 2	12	0	12	0.45	5.42	100
	SCoT 11	8	0	8	0.42	3.36	100
	SCoT 13	5	3	8	0.43	2.15	62
	SCoT 20	2	0	2	0.20	0.48	100
	SCoT 22	7	2	9	0.41	2.93	77
	SCoT 28	10	0	10	0.42	4.21	100
	SCoT 35	5	0	5	0.35	1.79	100
	SCoT 36	9	1	10	0.43	3.92	90

TB – total number of amplified bands; PB – polymorphism bands; PPB – percentage of polymorphism bands

Polymorphism percentage ranged from 62 (SCoT13) to 100 (SCoT1, SCoT2, SCoT11, SCoT20, SCoT28, SCoT35) with an average of 92 for the nine primes used. Amplification profile obtained with SCoT13 is presented in Figure 1. SCoT20 primer with 0.2 and SCoT1 and SCoT2 primers with

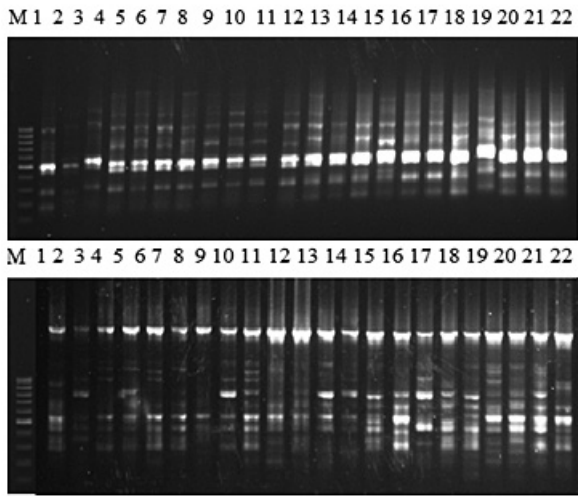

Fig. 1. Amplification profile obtained with KNOX-03 and SCoT13 primers detected in gerbera genotypes

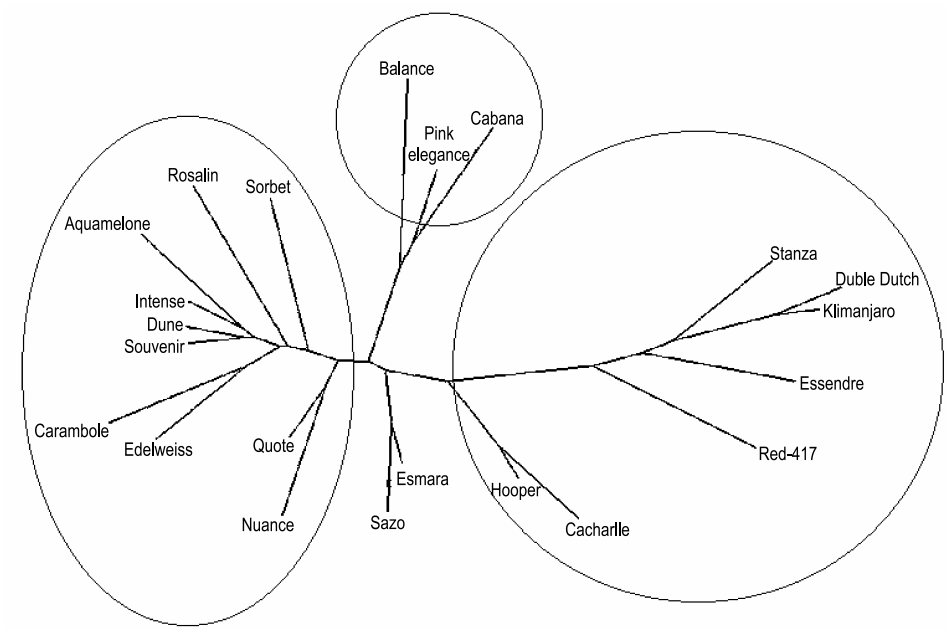

Fig. 2. Dendrogram of the 22 gerbera cultivars based on the dissimilarity matrix developed using SCoT markers

0.45 showed the lowest and highest PIC values among all primers, respectively. Average of PIC was 0.39 per locus. Marker Index (MI) ranged from 0.48 (SCoT20) to 6.4 (SCoT1) – Table 3. The similarity value of the 22 genotypes based on Jaccard's coefficient varied between 0.14 and 0.85. The highest genetic diversity was between Dune and Duble Dutch genotypes. The NJ clustering algorithm from SCoT analysis grouped the 22 genotypes into three clusters. Cluster I contained seven genotypes, cluster II included 11 genotypes, and cluster III included three genotypes (Figure 2). The cluster patterns obtained by SCoT primer is shown in Figure 2. The maximum and minimum number of fragments belonged to SCoT1 (14 bands) and SCoT20 (2 bands), respectively. The amplified bands ranged between 100 and 2500.

CCDP analysis

CDDP primers produced a total of 70 fragments of which 63 fragments were polymorphic bands. The average number of polymorphic bands was 7 per primer ranging from 3 (WRKY-R3) to 10 (MYB-02, WRKY-R1, and ERF1). The polymorphism percentage ranged from 57 to 100 % with a mean of 90%, showing a high polymorphism level. The mean value of PIC was 0.4 per locus which ranged from 0.28 to 0.44 (Table 3). Marker Index (MI) ranged from 0.85 (WRKY-R3) to 4.39 (ERF1) with a mean of 2.94 (Table 3). The similarity value of the 22 genotypes based on Jaccard's coefficient varied between 0.14 and 0.91. The highest genetic diversity was between Balance and Quote genotypes. Neighbor-Net cluster analysis based on CDDP grouped gerbera cultivars into three clusters (Figure 3). Clusters I, II, and III contained four, eight, and seven members, respectively. CDDP clusters had a relatively similar grouping pattern with those obtained by SCoT markers. Amplification profile obtained with KNOX-03 marker is presented in Figure 1. The amplified bands ranged between 150 and 2500.

Correlation between the similarity values using two marker systems

Cophenetic correlation estimated for the two marker systems were 0.86 and 0.86 for SCoT and CDDP, respectively, indicating a good fit for clustering. Positive correlation was observed between the two marker types using mantel test. The correlation coefficient (r) between SCoT and CDDP (significant $P > 0.01$) was 0.73, indicating a high similarity in DNA sequence variation at primer binding.

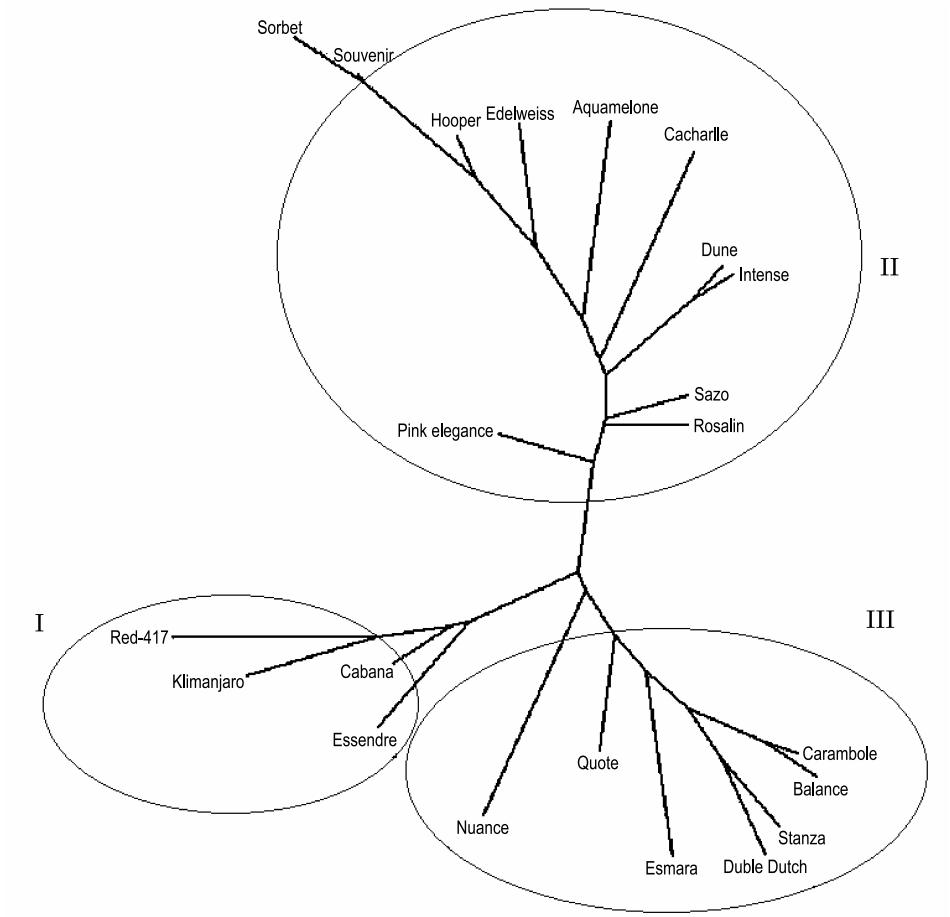

Fig. 3. Dendrogram of the 22 gerbera cultivars based on the dissimilarity matrix developed using SCoT CDDP markers

Discussion

Identification of genetic diversity and classification of genetic resources (germplasm) are important and essential for breeding and management of plant genetic resources. Gerbera is one of the most important cut flowers worldwide therefore breeding of this flower is necessary. Genetic diversity of gerbera is the first step toward flower improvement and researchers utilize molecular markers to assess genetic diversity among horticultural crops. The SCoT and CDDP marker techniques were employed in the present study for many reasons. Both genetic diversity and fingerprinting studies are of useful tools which enable plant breeders to make better decisions regarding selection of germplasm to be used in hybridization.

Our results demonstrated that CDDP and SCoT markers can be used as reliable techniques for detecting levels of DNA polymorphism and genetic relationships in gerbera. Conserved DNA regions sharing the same priming site, but differing in their genomic distribution may yield a large number of easily detectable length polymorphisms.

SCoT markers have many advantages including easy to work with in laboratory conditions, dominant markers, informative, inexpensive, and higher reproducibility compared to arbitrary markers such as RAPD (POCZAI et al. 2013). SCoTs can be utilized either in isolation or in combination with other techniques to evaluate genetic diversity and to obtain reliable information about population processes and structure across different plant families (ABDIN et al. 2017).

In the current study, for the first time two different markers, SCoT and CDDP, were used for analyzing the genetic diversity within a set of 22 diverse gerbera genotypes to know if these marker systems can be effectively used in breeding programs. Our study suggested that SCoT and CDDP markers can produce a highly considerable polymorphism and diversity in the studied gerbera cultivars which is in agreement with those reported in rose and anthurium (SAIDI et al. 2017, SAIDI et al. 2018). Using the combined markers, our findings showed the presence of a significant polymorphism and revealed a high level of variability in surveyed gerbera cultivars which is in agreement with those reported by Poczaie (2013) and Saidi et al. (2018).

CDDP can be used as functional markers (FM) and amplifies conserved DNA regions sharing the same priming site. This marker generated detectable length polymorphisms by high annealing temperature with high reproducibility and efficiency (POCZAI et al. 2013). It has been reported that polymorphism index content (PIC) in anthurium was calculated 0.42 and 0.37 for SCoT and CDDP, respectively. According to our findings, PIC for SCoT and CDDP markers was estimated 0.39 and 0.4, respectively. Average Marker index (MI) for SCoT and CDDP was calculated 3.40 and 2.45, respectively (SAIDI et al. 2018). There has been a report on the analysis of the genetic diversity among landraces and improved safflower genotypes collected from different geographical locations using gene-targeted (SCoT, CDDP, and CBDP) molecular markers. High level of polymorphism using these markers showed that they can be effective in determining the genetic diversity among safflower genotypes (TALEBI et al. 2018).

There have also been attempts to combine other primers in CDDP reactions to amplify polymorphic regions representing DNA stretches between two identical or very similar conserved primer binding sites (HAJIBARAT et al. 2015). Since both markers exhibited a relatively similar

and high PIC and MI values it would be safe to reason that both can be equally valuable in assessing genetic diversity or diagnostic fingerprinting in gerbera. Thus, it can be suggested that the use of SCoT and CDDP markers are more useful for estimating the genetic diversity in gerbera breeding programs.

Although the level of diversity for the two marker systems was nearly equal, however, we predict that the source of recognized diversity might be different. Therefore, each technique amplifies various regions of the genome. Dendograms obtained by SCoT and CDDP could be explained by the similar nature of each maker, extent of polymorphism, number of loci, and specific regions of the genome (Gorji et al. 2011, Saidi et al. 2018). These findings are contrary to the findings reported in potato (Gorji et al. 2011), wheat (Hamidi et al. 2014), and chickpea (Paksereshт et al. 2013).

Mantel correlation coefficient test showed a highly positive correlation between SCoT and CDDP matrices, indicating a stable relationship between genetic distances for both marker systems. This correlation may have been related to similarity in DNA sequence variations at primer binding sites between the SCoT and CDDP markers. The low narrow genetic base and genetic diversity in gerbera leads to slow genetic improvement of this plant. The selection of genotypes for genetic diversity was primarily based on different polymorphisms as obtained by molecular markers. Our findings showed that CBDP and SCoT techniques were highly reproducible and efficient and can be used as powerful tools for assessing genetic diversity among gerbera genotypes. This study showed that SCoT and CDDP techniques are eligible tools to detect the genetic diversity and genetic association of gerbera germplasm. Thus, it is suggested that the use of these markers are appropriate for crop improvement programs particularly in assessing genetic diversity, genotype identification, bulk population, and QTL mapping. SCoT and CDDP markers are based on functional regions of genome and their utilization are easy and not time-consuming. Finally, it is suggested that we need to increase the genetic base of gerbera germplasm through introduction, distance hybridization, and even mutagenesis for effective implication of markers.

This paper is the first report on the use of SCoT and CDDP markers on gerbera. Our results demonstrated that CDDP and SCoT markers can be used as reliable techniques for detecting levels of DNA polymorphism and genetic relationships in gerbera. In this study, the two different gene-targeted molecular markers, i.e. SCoT, and CBDP, adopted to study the genetic diversity among gerbera genotypes from different sources demonstrated that they have advantages over the use of dominant random markers (such as ISSR, RAPD, DAFs, and AFLP), as these markers reveal

genetic diversity from the genic region in the genome and this functional diversity can be used in any species (PALIWAL et al. 2013, HEIKRUJAM et al. 2015, ANDERSEN and LUBBERSTEDT 2003). These results demonstrate that SCoT markers are useful for cultivar identification and genetic diversity analyses of gerbera cultivars. This genetic information will support germplasm management and cultivar improvement in gerbera.

Conflict of interest

The authors declare that they have no conflict of interest.

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