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Comparative assessment of ISSR, DAMD and RAPD markers for evaluation of genetic diversity of gerbera (*Gerbera jamesonii* Bolus ex Hooker f.) cultivars

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Abstract: Genetic diversity is the best way to improve available genetic resources for breeding programs in gerbera. In present study, genetic diversity of 22 gerbera cultivars was investigated using inter-simple sequence repeat (ISSR), directly amplified minisatellite DNA (DAMD), and Random Amplified Polymorphic DNA (RAPD) markers. Average polymorphism information content (PIC) for ISSR, DAMD and RAPD markers was 0.40, 0.41 and 0.40, respectively. Cluster analysis for ISSR, DAMD and RAPD divided the cultivars into three distinct clusters. The comparative analysis of the three markers (ISSR, DAMD and RAPD) showed that DAMD had superiority over RAPD and ISSR in characterization of genetic diversity in *Gerbera*. To our knowledge, this is the first report of a comparison of performance among DAMD, ISSR and RAPD techniques on a set of gerbera genotypes. Overall, our results showed that DAMD markers well represented different genotypes of gerbera diversity.

Key words: *Gerbera*; DAMD; genetic diversity; ISSR; DAMD

Primerjalno preverjanje ISSR, DAMD in RAPD markerjev za vrednotenje genetske raznolikosti sort gerbere (*Gerbera jamesonii* Bolus ex Hooker f.)

Izvleček: Genetska raznolikost je izhodišče razpoložljivih genetskih virov v žlahniteljskih programih gerbere. V študiji smo genetsko raznolikost 22 sort gerbere preučevali z označevalci ISSR (inter-simple sequence repeat, regije med mikrosateliti), DAMD (direct amplified minisatellite DNA, pomnoženi minisateliti) in RAPD (Random Amplified Polymorphic DNA, naključno pomnožena polimorfna DNA). Poprečna informacijska vrednost polimorfizma (PIC) je bila za ISSR, DAMD in RAPD markerje 0,40; 0,41 in 0,40. Klasterska analiza ISSR, DAMD in RAPD markerjev je sorte razdelila v tri različne skupine. Primerjalna analiza treh markerjev (ISSR, DAMD in RAPD) je pokazala, da so imajo DAMD markerji prednost pred RAPD in ISSR za določitev genetske raznolikosti pri gerberi. Pričujoča raziskava je ena prvih, ki primerja učinkovitost DAMD, ISSR in RAPD markerjev na izbranem naboru genotipov gerber. Naši rezultati so pokazali, da so markerji DAMD najbolje prikazali raznolikost genotipov gerbere.

Ključne besede: *Gerbera*; DAMD; genetska raznolikost; ISSR; DAMD

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1 INTRODUCTION

Gerbera (*Gerbera jamesonii* Bolus ex Hooker f.)) belongs to the Compositae family and is one of the top five cut flowers in the world in terms of production and consumption, which has a great economic value in the international flowering industry (Aghdam et al., 2019). *Gerbera* (Compositae), is native to tropical regions of South America, Africa and Asia (Danaee et al., 2011). It is a diploid species with somatic chromosome number of 2n = 50. *Gerbera* is commercially important and it is considered a tender perennial plant. Gerbera was introduced to China in the 1980s and has been used as a model organism in surveying flower formation (Yang, 2012). Evaluation of genetic diversity and genetic relationships among cultivars are necessary tools for future breeding and improvement programs. Selection is essential for germplasm breeding and is effective only when the visible variation in the population is highly heritable (Kumari et al., 2011). Detection of genetic diversity and classification of genetic resources (germplasm) are important and essential strategies in breeding and preservation of plant genetic resources. Breeding of gerbera is based on crossing among cultivars and thereby phenotypic selection of new and improving progeny, followed by producing new cultivars. Molecular markers are an appropriate tool for measuring the diversity of plant species. Important factors such as low assay cost, affordable hardware, throughput, convenience and ease of assay development, make this technique an important aid to breeding and cultivar development (Rafalski, 2002). ISSRs can be targeted towards sequences, which are reported to be abundant in the genome and can overcome the technical difficulties of RFLP. In recent years, many new alternative and promising marker techniques have been developed in line with the rapid growth of genomic research. Minisatellite DNAs are tandemly repeated regions of genomes and are used in a procedure denoted directed amplification of minisatellite-region DNA (DAMD). This technique utilized help effectively in genetic diversity analysis in gerbera. Genetic diversity in the gerbera has been reported using diverse molecular markers such as RAPD (Prajapati et al., 2014), and EST-SSR (Gong et al., 2010), ISSR (Li et al., 2004). In the present study, RAPD, ISSR and DAMD markers were used as useful tools for genetic variability analysis to provide more comprehensive insight regarding the genetic relationships and germplasm management of the cultivars utilized in this study (Kumar et al., 2011). Here, we report for the first time the use of the DAMD marker-based technique for surveying genetic diversity in gerbera cultivars. The objectives of the present research were to evaluate genetic diversity of gerbera using three markers namely ISSR, DAMD, and RAPD. The efficiencies of these markers were also evaluated and compared for diagnostic fingerprinting of the gerbera cultivars.

2 MATERIALS AND METHODS

The plant material used in this study included twenty-two cultivars of *G. jamesonii* were collected from the Research Centre for Plants and Flowers (RCPF), Mahallat, Iran. Names of the cultivars are given in Table 1.

Genomic DNA was extracted from young leaves of plants according to the modified CTAB method as described by Lassner et al. (1989). The purified total DNA was quantified by agarose-gel electrophoresis using a known concentration of uncut λ DNA as a standard. A set of 30 RAPD primers were procured of which 17 primers gave clear and polymorphic patterns. The polymorphic primers were then used for further analysis of 22 cultivars (Table 2). PCR amplifications were performed in 25 µl reactions containing 30 ng sample DNA, 2.5 µM primer, 200 µM of each dNTP, 1.5–2.5 mM $MgCl_2$ (Magnesium chloride) and 1.5 unit of Taq DNA polymerase (Cinnagene, Iran). Thermal cycling included 3 min at 94 °C followed by 35 cycles of denaturation at 93 °C for 45 s, annealing at optimum temperature for 45 s, extension at 72 °C for 90 s, and a final extension cycle at 72 °C for 10 min. PCR products were separated on 1.5 % agarose gels, stained with ethidium bromide and scored for the presence or absence of bands.

Six ISSR and eight DAMD primers were selected for final amplification (Table 2). The amplification was performed in a thermal cycler (Eppendorf, Germany) with the following conditions: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, an-

Table 1: Names of the studied cultivars in this research

Sample NO.	Genotype	Sample NO.	Genotype
1	Rosalin	12	Cacharlle
2	Sorbet	13	Hooper
3	Souvenir	14	Nuance
4	Dune	15	Quote
5	Intense	16	Esmara
6	Aquamelone	17	Sazo
7	Edelweiss	18	Pink Elegance
8	Carambole	19	Essendre
9	Balance	20	Cabana
10	Stanza	21	Klimanjaro
11	Double Dutch	22	Red-417

Table 2: RAPD, ISSR, and DAMD primers used for amplification

No.	Name primer	Seq (5'-3')	No.	Name primer	Seq (5'-3')
ISSR			RAPD		
1	UBC828	TGTGTGTGTGTGTGTGA	17	OPE-10	CACCAGGTGC
2	UBC822	TCTCTCTCTCTCTCTCA	18	OPF-02	GAGGATCCCT
3	UBC801	ATATATATATATATATT	19	OPG-19	GTCAGGGCAA
4	UBC874	CCCTCCCTCCCTCCCT	20	OPK-17	CCCAGCTGTG
5	UBC816	CACACACACACACACAT	21	OPL-08	AGCAGGAGGA
DAMD			22	OPM-03	GGGGGATGAG
7	URP2F	GTGTGCGATCAGTTGCTGGG	23	OPN-04	GACCGACCCA
8	URP9R	AGGACTCGATAACAGGCTCC	24	OPQ-11	TCTCCGCAAC
9	URP25F	GGCAAGCTGGTGGGAGGTAC	25	OPR-10	CCATTCCCCA
10	URP30F	TACATCGCAAGTGACACAGG	26	OPR-17	CCGTACGTAG
11	URP13R	AATGTGGGCAAGCTGGTGGT	27	OPAA-04	AGGACTGCTC
12	URP17R	GATGTGTTCTTGGAGCCTGT	28	OPAF-10	GGTTGGAGAC
13	URP 6R	GGACAAGAAGAGGATGTGGA	29	OPAD-06	AAGTGCACGG
14	URP2F	AAGAGGCATTCTACCACCAC	30	OPAE-05	CCTGTCAGTG
RAPD					
15	OPB-07	GGTGACGCAG			
16	OPD-06	ACCTGAACGG			

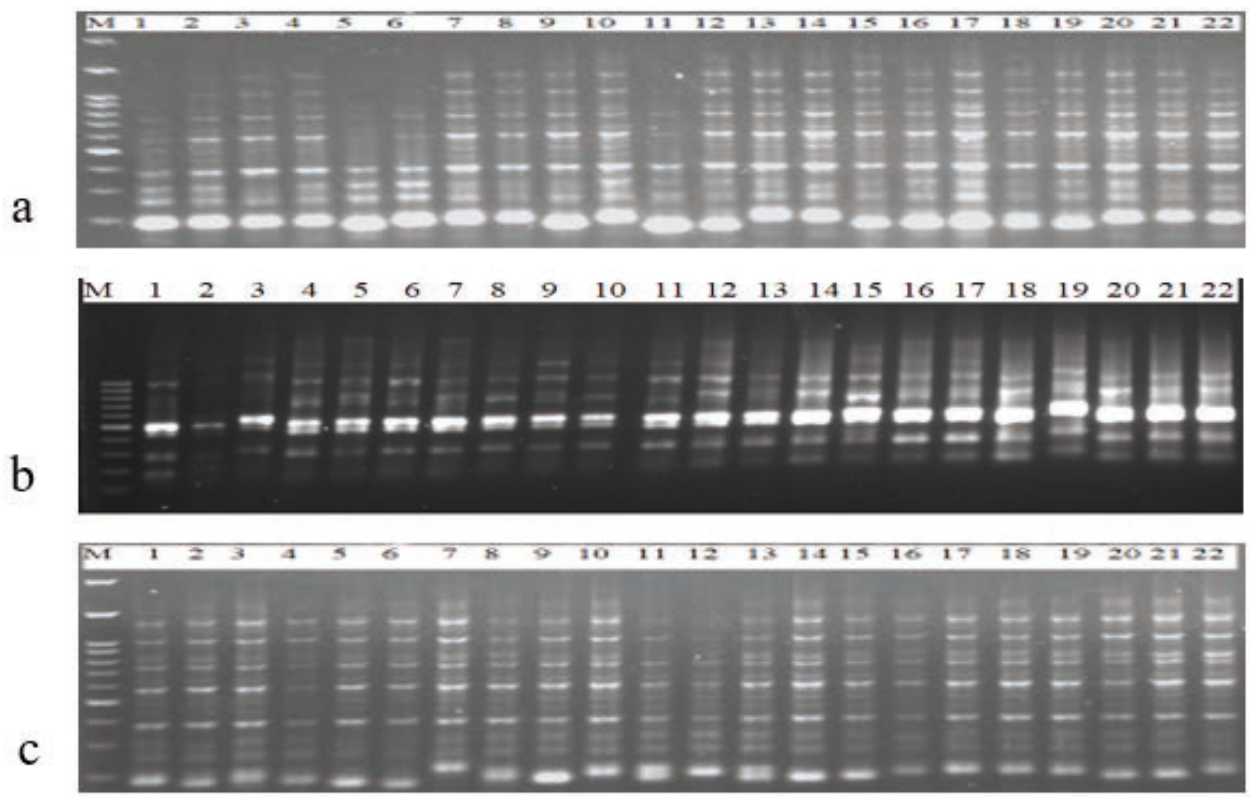

Fig. 1: The RAPD pattern obtained with OPZ-17 primer (a), the ISSR pattern obtained with UBC822 primer (b), and the DAMD pattern obtained with URP17R (c)

Table 3: DAMD, ISSR, and RAPD markers used for genetic diversity analysis

primer	No. of polymorphic bands	No. of monomorphic bands	No. of amplified bands	PIC value	Polymorphism (%)	MI
ISSR						
UBC828	4	0	4	0.33	100	1.34
UBC822	9	0	9	0.44	100	3.96
UBC801	5	2	7	0.38	100	1.93
UBC874	10	0	10	0.44	100	4.94
UBC816	7	0	7	0.42	71	2.97
DAMD						
URP2F	7	0	7	0.39	100	2.76
URP9R	10	0	10	0.43	100	4.39
URP25F	5	0	5	0.38	100	1.93
URP30F	7	0	7	0.41	100	2.93
URP13R	10	1	11	0.43	90	4.32
URP17R	9	0	9	0.43	100	3.92
URP 6R	6	0	6	0.40	100	2.41
RAPD						
OPB-07	9	0	9	0.42	100	3.86
OPD-06	8	0	8	0.35	100	2.87
OPE-10	9	1	10	0.39	90	3.56
OPF-02	11	1	12	0.43	91	4.8
OPG-19	10	0	10	0.44	100	4.47
OPL-08	7	1	8	0.44	87	3.01
OPZ-17	13	0	17	0.48	100	6.71
OPM-03	7	1	8	0.39	87	2.75
OPN-04	7	0	7	0.41	100	2.87
OPQ-11	6	1	7	0.39	85	2.38
OPR-10	5	0	5	0.37	100	1.86
OPR-17	7	1	8	0.44	87	3.01
OPAA-04	7	0	7	0.41	100	2.87
OPAF-10	8	0	8	0.42	100	3.42
OPAD-06	6	0	6	0.36	100	2.18
OPAE-05	2	0	2	0.24	100	0.48

nealing at 48 °C (for ISSR analysis) and 50 °C (for DAMD analysis) for 1min each, extension at 72 °C for 2 min and final extension at 72 °C for 7 min. The PCR products obtained were separated on 2 % agarose gel using 1 × TBE buffer at a constant voltage of 100 V for one hour. The gel stained with ethidium bromide and visualized using a gel documentation.

The amplified RAPD, ISSR and DAMD fragments obtained were scored for presence (1) or absence (0) of bands. Tree construction following an NJ tree using a similarity matrix was performed through Splits Tree. The dissimilarity matrix thus obtained was subjected to cluster analysis using the un-weighted neighbor-joining analyses (UNJ), followed by bootstrap analysis with 1,000 permutations to obtain a dendrograme for all 22 genotypes. Mantel statistic was used to compare the similarity

Fig. 2: Dendrogram of the 22 gerbera genotypes based on the dissimilarity matrix developed using ISSR markers

matrices as well as the dendrogrames produced by the ISSR, DAMD, and RAPD techniques. All these procedures were performed by appropriate routines in NTSYS-pc version 2.0. Polymorphic information content (PIC) values were calculated for each ISSR, DAMD, and RAPD primers according to the formula: $PIC = 1 - \sum(Pij)2$; where Pij is the frequency of the i^{th} (frequency of the ith pattern) pattern revealed by the j^{th} (primer summed across all patterns) primer summed across all patterns revealed by the primers. The Mantel test of significance (Mantel 1967) was also used to compare each pair of similarity matrices produced.

3 RESULT AND DISCUSSION

The 17 primers produced a total of 143 reliable fragments of which 138 were polymorphic. The number of fragments generated by these RAPD primers was found to range from 2 to 15 bands. OPZ-17 primer produced the maximum number of polymorphic bands and OPAE-05 primer generated the minimum number of polymorphic bands. The RAPD pattern obtained with OPZ-17 primer is shown in Fig1a. The percentage of polymorphism ranged from 85 % for OPQ-11 to 100 % for OPN-04, OPR-10, OPAF-10, OPZ-17, OPAE-05, OPA-02, OPK-07, OPL-08, OPG-19, OPB-07, OPAD-06, and OPD-06 with an average of 96 % polymorphism per primer. The polymorphism information content ranged from a 0.44 (OPG-19, OPL-08, OPZ-17, and OPR-17) to 0.24 (OPAE-05) with an average of 0.40, indicating hypervariability among the individuals studied. The marker index (MI) for RAPD was the highest for the primer OPZ-17 (6.71) and the lowest for the primer OPZ-17 (0.48). An average MI of 2.52 per primer was observed. Cluster analysis showed similar grouping pattern.

A total 10 ISSR primers were screened of which five primers showed polymorphic bands. A total of 57 polymorphic bands were detected amongst 22 gerbera cultivars using 5 ISSR markers. Average polymorphism percentage was 94 % across all cultivars. The number of products generated per primer was found to range from 4 (UBC 828) to 10 (UBC 874) bands. The ISSR pattern obtained with UBC822primer is shown in Fig1b. Polymorphism information content (PIC) values ranged from 0.33 to 0.44, with an average value of 0.40 per prim-

Fig. 3: Dendrogram of the 22 gerbera genotypes based on the dissimilarity matrix developed using RAPD markers

er (Table 3). Cluster analysis grouped *Gerbera* cultivars into three distinct clusters (Fig. 2). Cluster I and II each contained seven cultivars. The marker index was found to be the highest for primer UBC874 (4.94) and the lowest for the primer UBC828 (1.34) with an average MI of 3.03 per primer. Cluster III showed similar grouping patterns with those obtained by RAPD data.

Cluster I and II included 8 and 7 cultivars, respectively. Cluster III contained 3 cultivars (Fig 3). Seven primers generated a total of 55 bands of which 54 bands were polymorphic. The number of amplified products generated by DAMD primers were in the range of 5–10 bands. Primers URP13R and URP9R generated the maximum (10 bands) and primer URP25F generated the minimum (5 bands) number of amplicons. Average polymorphism percentage was 99 % across all cultivars. PIC values ranged from 0.38 to 0.43, with an average value of 0.41 per primer (Table 4). The DAMD pattern obtained with URP17R primer is shown in Fig. 1c. Based on un-weighted neighbour-joining method, all 22 gerbera varieties were grouped into three distinct clusters (Fig. 3). The estimates of MI were found to be the highest for primer URP9R (4.39) and the lowest with the primer URP25F (1.93) with an average MI of 3.24 per primer. Cluster I contained seven cultivars, cluster II included 11 cultivars, and cluster III included four cultivars showing relatively a similar grouping patterns with clusters III and IV of ISSR and cluster I of DAMD markers (Fig 4).

Our study using three marker systems suggested the presence of a significant polymorphism and revealed high level of variability in surveyed gerbera genotypes which agrees with those reported by Prajapati (2014). This is the first research to utilize RAPD, ISSR and DAMD molecular markers to examine the extent of genetic variability among germplasm of gerbera. In the current study, three different molecular markers, ISSR, DAMD, and RAPD were employed for the analysis of the genetic variability of a set of 22 gerbera cultivars. The results of our study suggests that these markers can be used in breeding programmes to reliably distinguish studied gerbera varieties. A comparison of level of polymorphism and effective performance of ISSR, DAMD, and RAPD showed that each of the three markers can be detected genetic relationships and discriminating efficacy within cultivars. All of the three markers were used to show high level of polymorphism. Previous studies also revealed that DAMD

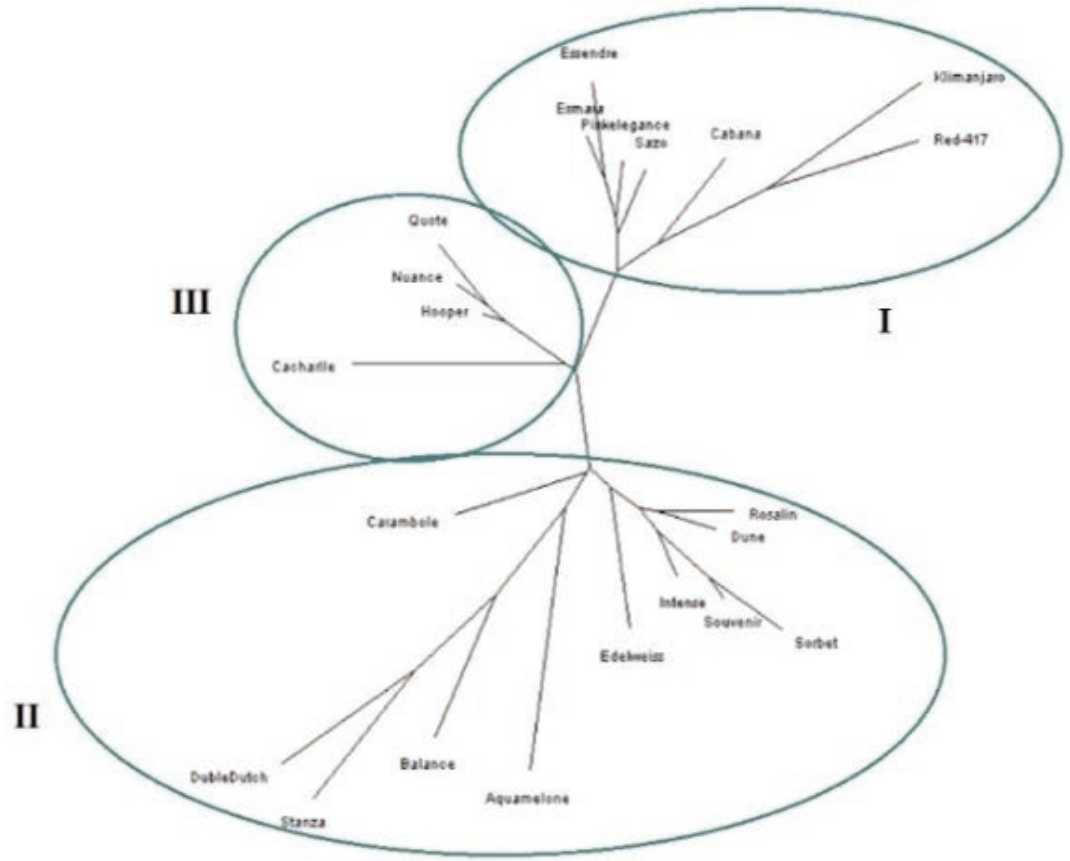

Fig. 4: Dendrogram of the 22 gerbera genotypes based on the dissimilarity matrix developed using DAMD markers

techniques were able to provide more reliable diversity information compared to RAPD or ISSR techniques (Pakseresht et al., 2013) and are useful as tools for studying the genetic diversity of different plant germplasm. In comparison with those studies, we observed that the average polymorphism rate for RAPD, ISSR, and DAMD markers in gerbera cultivars were relatively high. ISSR and DAMD showed higher polymorphism percentage than RAPD but PIC in three markers equal. These results suggested the presence of a considerable polymorphism in two markers at studied and revealed a high potential of genetic diversity in the existing gerbera germplasm. Mantel coefficient correlation test showed higher positive correlation between ISSR and DAMD metrices, indicating a consistent relationship between genetic distances from both marker systems. The correlation coefficient (r) was 0.73 between ISSR and RAPD, 0.80 between DAMD and ISSR, and 0.77 (significant $p > 0.01$) between RAPD and DAMD. All three molecular marker types showed positive but significant correlation with each other. Based on Mantel test results, the rate of genetic diversity for genotypes was in ISSR and DAMD approximately equal but RAPD was less than two markers. We predict that the source of detected diversity is various, as each technique targets different regions of the genome. Results of mantel coefficient correlation test showed higher positive correlation between ISSR and DMAD metrices, indicating a consistent relationship between genetic distances from both marker systems. This higher correlation may have been attributable to similarity in DNA sequence variation at primer binding sites between the ISSR and DAMD techniques. It is interesting to note that all three (ISSR, DAMD, and RAPD) datasets showed high levels of correlation. This is not surprising as these markers are known to target different genomic fractions involving repeat and/or unique sequences, which may have been differentially evolved or preserved in due course of natural or human selection. These techniques were more informative and effective than the previously used molecular methods to study variation in gerbera, such as SSR (Hajibarat et al., 2014), and RAPD markers (Feghhi et al., 2014, Prajapati et al., 2014, Saidi et al., 2018). Discordance between dendograms obtained by ISSR and DAMD with RAPD could be explained by the different nature of each technique, region coverage of genome by each marker, level of polymorphism and the number of loci.

Our results substantiate the previous reports by clustering genotypes using different marker systems in chickpea (Pakseresht et al., 2013), and Lenti (Seyedimoradi and Talebi 2014). Although the level of diversity for the three marker techniques was approximately equal, we offered that there are several possible explanations for such results: some of them linked with stricter of different molecular markers that designed from different regions of genome. Comparison with MI between ISSR, DAMD, and RAPD shows that DAMD has higher marker rate than RAPD and ISSR. The MI, which can be a resolving power to distinguish among different accessions (Khodadadi et al., 2011), was different in three marker markers (Table 3). Our study revealed that the resolving power of DAMD markers is higher than ISSR primers. Information about current genetic diversity permits the classification of our available germplasm into various/heterotic groups, which is particularly important to hybrid/cross-breeding programs in gerbera. DAMD markers are usually reproducible, while primer length and annealing temperature are not the sole factors determining reproducibility (Saidi et al., 2017).

In conclusion, DAMD marker analysis was successfully developed to evaluate the genetic relationships among the gerbera cultivars. Polymorphism percentage revealed by DAMD was so abundant and could be used for molecular genetics study of the gerbera cultivars, providing high-valued information for the management of germplasm, improvement of the current breeding strategies, and conservation of the genetic resources of *Gerbera* species.

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