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Full Length Research Paper

Exploitation of random amplified polymorphic DNA (RAPD) and sequence-related amplified polymorphism (SRAP) markers for genetic diversity of saffron collection

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Saffron is the most expensive spice in the world. Considering the importance of germplasm characterization and evaluation particularly in saffron, the present study was formulated with an objective to access the structure of genetic diversity among the accession of saffron at molecular levels. Twenty six primers from Operon Technologies were used in random amplified polymorphic DNA (RAPD) analysis, thirty primers were screened using sequence-related amplified polymorphism (SRAP) method were assayed in six clone of *Crocus sativus* from six center of production of saffron in Iran. The RAPD primers developed 95 robust loci, which revealed 56% polymorphism and 75 SRAP markers generated 43% polymorphism. Genetic diversity parameters for RAPD and SRAP along with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering based on Jaccard coefficient were estimated with a view to assess efficiency of the marker system in saffron. Accessions were classified into four clusters using both methods, which supplied molecular evidences for the exploit of this rich source in Iran. RAPD markers were almost as efficient as SRAP markers with regards to detection of polymorphism, number of loci scored and polymorphism information content (PIC) values. Therefore saffron is not monomorphic and we can find diversity for breeding purposes.

Key words: *Crocus sativus*, genetic differentiation, molecular marker.

INTRODUCTION

Medicinal and aromatic plants have increasing in importance to society continuously for the past 100 years. Saffron (*Crocus sativus* Linn.) is the most expensive spice in the world. It is made from the dried stigmas of the saffron flower, a triploid sterile (2n = 24; x = 8) plant that is vegetatively propagated by means of corms (Fernández, 2004); it produces no fertilizable gametes (Ghaffari, 1986) and is self-incompatible (Grilli-Caiola et al., 2001). Archeological and historical sources (Negbi, 1999; Tammaro, 1987) indicate that saffron is a very old cultivation dating back to 2500 to 1500 BC, probably originated in Iran, Asia Minor or Greece and later widespread in India, China, the Mediterranean basin and Eastern Europe. While the world's total annual saffron production is estimated in 205 tons per year, Iran is said to produce 80% of this total, that is, 160 tons. Khorasan province alone accounts for 46,000 ha and 137 tons of the aforementioned totals (Bagheri and Vesal, 2002; Fernández, 2004).

The taxonomy of *Crocus* is extremely complicated due to the lack of clear distinctive characters, wide range of habitats and heterogeneity of the morphological traits, and cytological data (Rubio-Moraga et al., 2009). Whether saffron has undergone modifications along its millenarian cultivation and whether it has one or more ancestors is still uncertain (Grilli-Caiola et al., 2004).

Phenotype differences, such as flowers with a larger

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Table 1. Accessions of *Crocus sativus*.

Rank	Genotype	Province	City	Latitude (N)	Longitude (E)	Altitude (M)
1	saGoAz	Golestan	Azad Shahr	37° 00′	55° 32′	1381
2	saFaEs	Fars	Estahban	29°08′	59° 03′	1767
3	saKrGo	Khorasan Razavi	Gonabad	34°21′	58° 41′	1150
4	saKjFr	Khorasan Jonobi	Ferdos	34°01′	58° 11′	1210
5	saKjGh	Khorasan Jonobi	Gaen	33°44′	59° 12′	1440
6	saKjBr	Khorasan Jonobi	Birjand	32°53′	59° 15′	1480

number of styles, branches and stamens, have been described by Piccioli (1932) in field. Estilai (1987) reported that new variants of saffron with increased number of stigmas in Iranian cultivation, maintaining 2n = 24, although the frequency of the rare types was only 1.2 × 10^{-6} flowers.

Morphological comparison of saffron flowers from corms obtained from different countries revealed some differences in flower colour intensity and the presence of lobed tepals in some accessions, other than differences in pollen size and viability (Grilli-Caiola et al., 2001).

Technological advances in DNA techniques were made available to opening new insights on taxonomic analysis (Grilli-Caiola et al., 2004). There are few articles that used molecular and biochemical approaches in an attempt to classify and clarify the systematic and phylogeny of this genus (Alavi-Kia et al., 2008; Beiki et al., 2010; Frello and Heslop-Harrison, 2000; Frizzi et al., 2007; Grilli-Caiola Caiola et al., 2004; Nørbæk et al., 2002; Peterson et al., 2008; Rubio-Moraga et al., 2009; Rubio-Moraga A et al., 2010; Seberg and Peterson, 2009; Sik et al., 2008). PCR-based approaches are in demand because of their simplicity and also because they can be carried out with only small quantities of sample DNA. Genetic diversity and relationships among species or populations is an important topic in genetics and plant breeding. No published reports are available about random amplified polymorphic DNA (RAPD) and sequence-related amplified polymorphism (SRAP) analysis and phylogenetic relationships of *Crocus* species in Iran except report of Beiki et al. (2010). The objective of the present study was to determine whether *C. sativus* is a monomorphic or polymorphic specie. The different molecular approaches used were based on PCR methods such as RAPD and SRAP, which have been widely used as markers (Xue et al., 2010; Budak et al., 2004; Sik et al., 2008).

MATERIALS AND METHODS

Six *C. sativus* populations were sampled from six important center of production of saffron in Iran; Birjand, Ghaen, Gonabad, Ferdos, Estahban and Azad-Shahr (Table 1). Three individuals representing each population were used for molecular study. Plant tissues were independently harvested, frozen in liquid nitrogen and stored at −80°C until required.

Genomic DNA extraction

Total genomic DNA was extracted from leaves following the CTAB procedure (Dellaporta et al., 1985) with slight modification analyzed and quantified by standard methods (Sambrook et al., 1989). For each accession, 50 to 100 mg of ground leaf tissue from a bulk ten plant was suspended in 1 ml of extraction buffer [50 mM EDTA, 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, and SDS %10]. The suspension was mixed well, incubated at 60°C for 15 min. from stock potassium acetate to added 300 µl, followed by chloroform-isoamyl alcohol (24:1) extraction, and precipitation with equal volume of isopropanol at -20°C. The pellet formed after centrifugation at low speed for 10 min was washed with 76% (v/v) ethanol. The DNA was then suspended in Tris EDTA buffer. After RNAse treatment, quantity and quality of extracted DNA were evaluated with a Nano-Drop® ND-1000 UV-Vis Spectrophotometer (Labtech International) and in a 0.8% (w/v) agarose gel stained with ethidium bromide. DNA content was diluted to a 25 ng/µl working solution. The DNA was stored at –20°C.

SRAP analysis

The SRAP technique consists of preferential amplification of ORFs by PCR with two primers, a forward primer of 17 bases and a reverse primer of 18 bases. The forward primers contain a fixed sequence of 14 bases rich in G and C in the 5′ end and three selective bases in the 3′ end. The first ten bases starting at the 5′ end are “filler” sequences of no specific constitution, followed by the sequence CCGG and then by three selective nucleotides at the 3′ end. Variation in these three selective nucleotides generates a set of primers sharing the same core sequence. The reverse primers consist of the same components as the forward primers with the following variations: the filler is followed by AATT instead of the CCGG sequence. Following the AATT sequence, three selective bases are added to the 3′ end of the primer. The only rules for construction of the forward and reverse primers are that they do not form hairpins or other secondary structures, and to have a GC content of 40 to 50%. Further, the filler sequences of the forward and reverse primers must be different from each other and can be 10 or 11 bases long (Budak et al., 2004; Ferriol et al., 2004; Gao et al., 2008; Li-Wang et al., 2008; Li and Quiros, 2001; Peng et al., 2008; Shu-Jing et al., 2006; Uzun et al., 2010; Xue et al., 2010).

In this assay, 30 different primer combinations were used, with five forward and six reverse primers previously described for analyzing genetic diversity in *Brassica* and other genera (Li and Quiros, 2001) (Table 2). Each 15 µl PCR reaction mixture consisted of 100 ng genomic DNA, 0.2 mMdNTPs, 2.5 mM$MgCl_2$, 15 pmol primer, 1.5 µl 10× Taq buffer, and 1 unit of Taq polymerase. Samples were subjected to the following thermal profile: 4 min of denaturing at 94°C, five cycles of three steps: 1 min of denaturing at 94°C, 1 min of annealing at 35°C, and 1 min of elongation at 72°C. In the following 35 cycles, the annealing temperature was increased to 50°C, with a final elongation step of 4 min at 72°C. Separation of

Table 2. Primer sequences used for SRAP.

SRAP primer	Sequence (5′ - 3′)	SRAP primer	Sequence (5′ - 3′)
ME-1 (forward)	TGAGTCCAAACCGGATA	EM-1 (reverse)	GACTGCGTACGAATTAAT
ME-2 (forward)	TGAGTCCAAACCGGAGC	EM-2 (reverse)	GACTGCGTACGAATTTGC
ME-4 (forward)	TGAGTCCAAACCGGACC	EM-4 (reverse)	GACTGCGTACGAATTTGA
ME-5 (forward)	TGAGTCCAAACCGGAAG	EM-5 (reverse)	GACTGCGTACGAATTAAC
		EM-6 (reverse)	GACTGCGTACGAATTGCA

Table 3. List of primers used for RAPD profiling.

RAPD primer	Sequence (5′ - 3′)	RAPD primer	Sequence (5′ - 3′)	RAPD primer	Sequence (5′ - 3′)
Opron M1	GTTGGTGGCT	Opron M12	GGGACGTTGG	Opron K15	CTCCTGCCAA
Opron M3	GGGGGATGAG	Opron M13	GGTGGTCAAG	Opron K20	GTGTCGCGAG
Opron M4	GGCGGTTGTC	Opron M14	AGGGTCGTTC	Opron N1	CTCACGTTGG
Opron M5	GGGAACGTGT	Opron M15	GACCTACCAC	Opron N10	ACAACTGGGG
Opron M6	CTGGGCAACT	Opron M16	GTAACCAGCC	Opron N20	GGTGCTCCGT
Opron M8	TCTGTTCCCC	Opron M18	CACCATCCGT	Opron G5	CTGAGACGGA
Opron M9	GTCTTGCGGA	Opron M19	CCTTCAGGCA	Opron G6	GTGCCTAACC
Opron M10	TCTGGCGCAC	Opron M20	AGGTCTTGGG		
Opron M11	GTCCACTGTG	Opron K6	CACCTTTCCC		

the amplified fragments was performed on 6% (w/v) polyacrylamide gels [acrylamide-bisacrylamide (28:2), TBE 1x] at 90 w during 2 h. The gels were stained with $AgNO_3$ for visualizing the SRAP fragments. The fragments between 300 and 900 base pair (bp) were visually scored as present (1) or absent (0).

RAPD analysis

The RAPD technique consists of preferential amplification of random sequences by PCR. In this assay, 26 different primer were used (Table 3). Each 25 µl PCR reaction mixture consisted of 50 ng genomic DNA, 0.2 mMdNTPs, 2 mM $MgCl_2$, 10 pmol primer, 2.5 µl 10x Taq buffer, and 1 unit of Taq polymerase. Samples were subjected to the following thermal profile: 4 min of denaturing at 94°C, forty-five cycles of three steps: 30 s of denaturing at 94°C, 1 min of annealing at 36°C, and 2 min of elongation at 72°C, with a final elongation step of 7 min at 72°C. Separation of the amplified fragments was performed on 1.2% (w/v) agarose gels, TAE 1x] at 80 V during 2 h. The gels were stained with ethidium bromide for visualizing the RAPD fragments. The fragments between 200 and 4000 base pair (bp) were visually scored as present (1) or absent (0).

Data collection and analysis

Molecular size (bp) of amplified DNA fragment was determined by the 100 bp ladder and λ *Hin*d III/ *Eco*RI double digested marker that was loaded in separate well of agarose gel for each gel. Amplified products were scored separately for each primer as present (1) or absent (0) to form a binary matrix. This binary data was used to calculate the Jaccard's Similarity coefficient (JS). Genetic distances between each pair of population were estimated as DS = 1 – JS. DNA fragment analyses and principal coordinate analysis (PCoA) are based on a similarity matrix using the Numerical Taxonomy Multivariate Analysis System (NTSYSpc2) software package (Rohlf, 1992). Clustering is based on a similarity matrix using the Completed Linkage method with the SAHN module of NTSYS-pc to show a phenetic representation of genetic relationships as revealed by the Jaccard's similarity coefficient (Sneath and Sokal, 1973). The profiles generated in different *C. sativus* populations were analyzed to compute polymorphic information content (PIC) using appropriate mathematical derivations of population studies (Smeath et al., 1997). A normalized Mantel statistic was calculated between cophentic matrix computed from marker based tree matrix and original similarity data (Mantel, 1967).

RESULTS

Molecular characterization of SRAP

The SRAP selected primers were based on previous reports of Li and Quiros (2001), Ferriol et al. (2003), Budak et al. (2004), Sun et al. (2006), Guo et al. (2006), Wang et al. (2008), Peng et al. (2008), Hao et al. (2008) and Xue et al. (2010). A total of 30 different combinations of primers were employed using five forward primers and six reverse primers (Table 2), ten combinations of primers produced well-defined and scorable amplification products, which showed polymorphisms in all accessions (Figure 1).

A total of 75 reproducible fragments were observed (Table 4a). Among them 32 fragment were polymorphic, ranging in size from 100 to 3000 bp. Between 4 and 17, fragments were amplified per primer combination, with an average of 8.3 bands. The highest and the lowest PIC

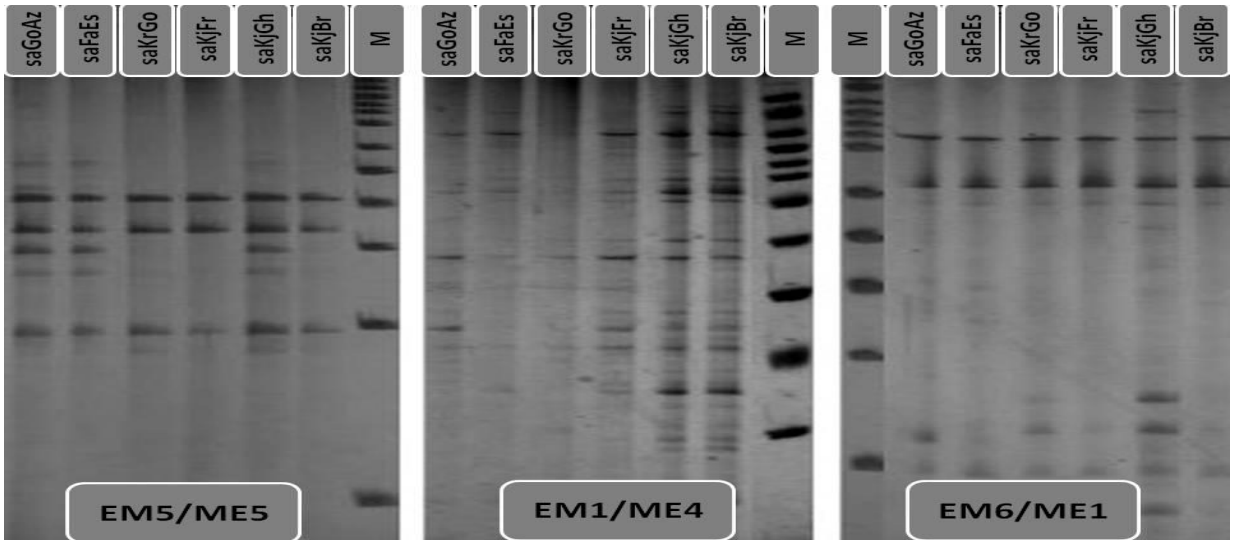

Figure 1. Amplification profile of SRAP with EM5/ME5, EM1/ME4 and EM6/ME1. Lane M is 1-kb ladder and other lanes represent different saffron genotypes as listed in Table 1.

Table 4. Number of polymorphic fragments using SRAP and RAPD markers.

SRAP primer (a)	Number of polymorphic bands	PIC	RAPD Primer (b)	Number of polymorphic bands	PIC
EM5/ME2	8	0.21	Opron M1	6	0.55
EM1/ME1	3	0.53	Opron M3	5	0.66
EM5/ME5	4	0.46	Opron M5	2	0.47
EM6/ME2	2	0.25	Opron M10	3	0.82
EM6/ME1	1	0.58	Opron M11	11	0.52
EM1/ME4	3	0.31	Opron M12	3	0.26
EM4-ME4	11	0.34	Opron M13	1	0.62
			Opron M16	8	0.23
			Opron M20	1	0.40
			Opron K6	5	0.23
			Opron G6	3	0.15
			Opron G12	5	0.33

scores were detected as 0.58 and 0.21 for primer combinations of EM5/ME2 and EM6/ME1, respectively (Table 4a). The genetic distance (GD) was computed for all 21 combinations of six accessions based on 32 polymorphic SRAP markers (Table 5a). The distance of six accessions ranged from 0.0556 (saKjFr vs. saKjBr) to 0.8947 (saKrGo vs. saKjGh) with an average of 0.6196 across all 21 pairs. For each accession maximum GD was observed with saKjGh.

The distinctiveness of the clusters identified in the Nei and Li distance (Nei and Li, 1979) and the UPGMA method complete dendrogram exhibited 4 distinct groups (Figure 3B). Normalized Mantel statistic showed significant correlation (r = 0.923). This implied a good fit for the cluster analysis performed.

The UPGMA dendrogram (Figure 2B) and PCoA Plot (Figure 4B) showed a clear separation between provinces and accessions were grouped according to their geographical origins. All grouping followed the same pattern in UPGMA dendrogram and PCoA plot. It is also evident from both graphs that the accessions originating from a common place tend to be in the same group. This has been true with the 3 accessions from Khorasan jonobi. Further, saKrGo from Khorasan razavi has maximum similarity to all genotypes of Khorasan jonobi.

Molecular characterization of RAPD

In this study, from 26 RAPD primers applied in the 6 *Crocus* accessions, 14 (54%) of them yielded polymorphic bands (Figure 3) producing a total of 95

Table 5. Jaccard similarity coefficient among saffron genotypes.

Genotype	saGoAz	saFaEs	saKrGo	saKjFr	saKjGh	saKjBr
(a): SRAP similarity matrix						
saGoAz	1.0000					
saFaEs	0.7727	1.0000				
saKrGo	0.1786	0.2000	1.0000			
saKjFr	0.2667	0.2963	0.5263	1.0000		
saKjGh	0.3636	0.4211	0.1053	0.3000	1.0000	
saKjBr	0.2581	0.2875	0.5000	0.9444	0.2857	1.0000
(b): RAPD similarity matrix						
saGoAz	1.0000					
saFaEs	0.6110	1.0000				
saKrGo	0.3148	0.4444	1.0000			
saKjFr	0.4630	0.5926	0.6667	1.0000		
saKjGh	0.4815	0.5370	0.5741	0.7222	1.0000	
saKjBr	0.4259	0.5556	0.5185	0.7407	0.7222	1.0000

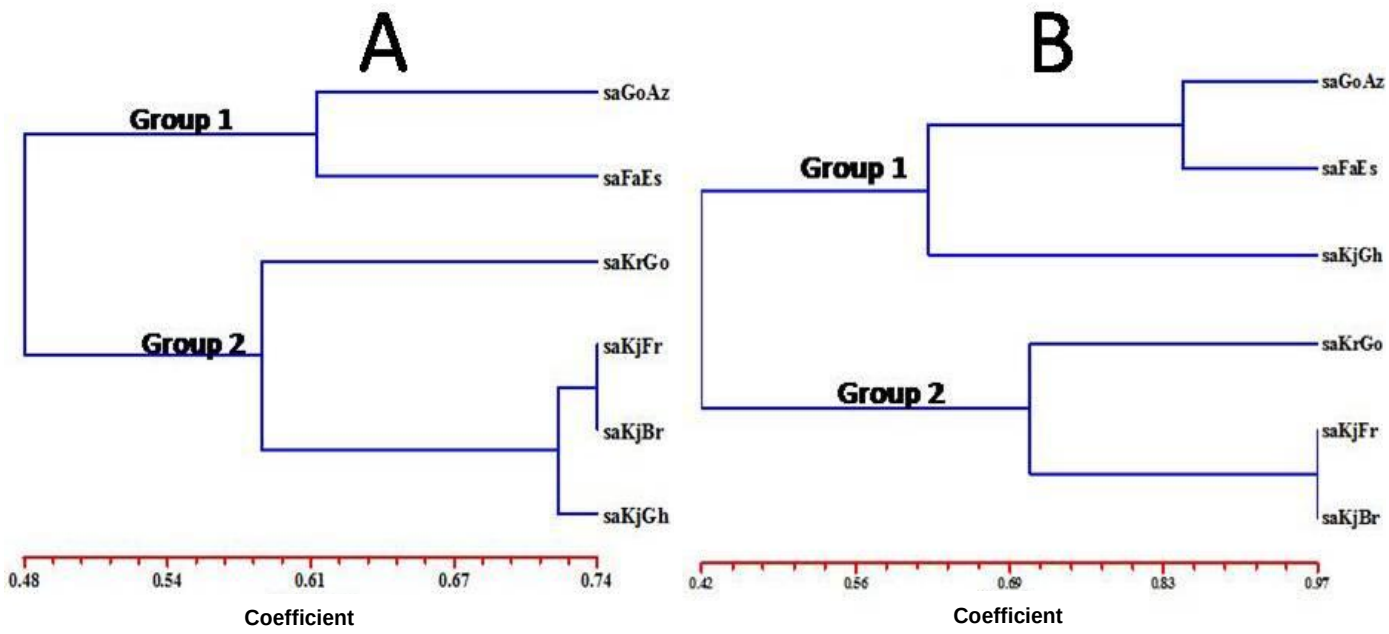

Figure 2. UPGMA dendrogram showing the relationships among 6 saffron genotypes (Table 1) with RAPD (A); SRAP (B).

reproducible fragments (Table 3). The remaining 12 primers produced either no polymorphic band or were excluded because of unclear banding patterns. Among them, 53 fragments were polymorphic, ranging in size from 200 to 4000 bp. Between 3 and 13 fragments were amplified per primer combination, with an average of 10 bands. The highest and the lowest PIC scores were detected as 0.82 and 0.15 for primer combinations of operonM10 and operonG6, respectively (Table 4b). The genetic distance based on RAPD data ranged from 0.2593 (saKjFr vs. saKjBr) to 0.6852 (saGoAz vs. saKrGo) (Table 5b) with an overall mean of 0.442. All the species revealed maximum GD with combination of saGoAz. The cluster analysis revealed similar four major groups like SRAP analysis (Figure 2A). UPGMA dendrogram grouped accessions based on geographical area. PCoA (Figure 4A) showed a clear separation between provinces and accessions were grouped according to their geographical origins. In these analyses, all grouping followed the same pattern. It is also evident

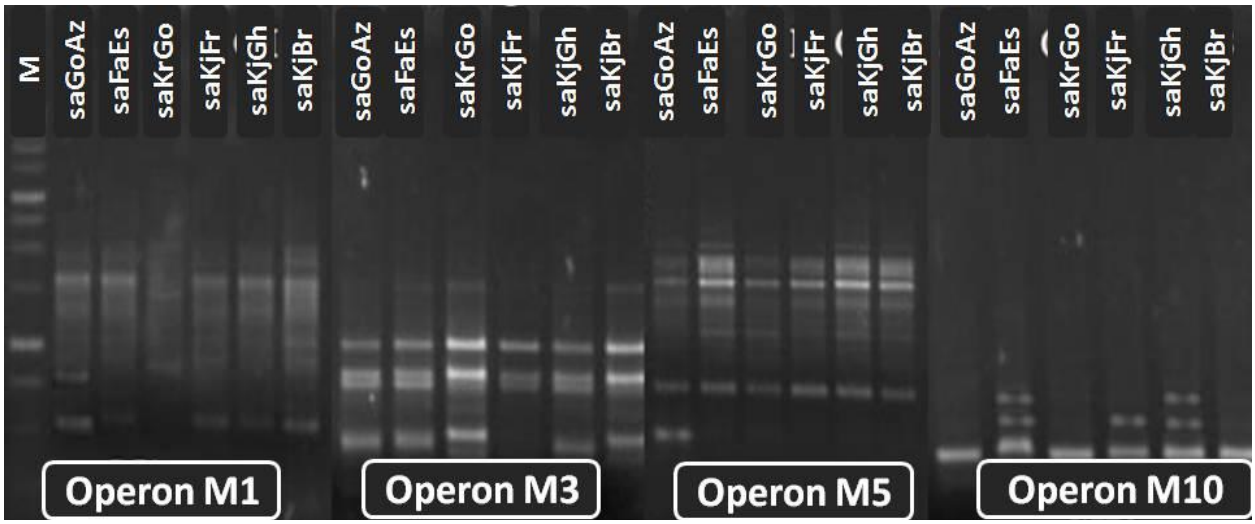

Figure 3. Amplification profile of RAPD with M1, M3, M5 and M10. Lane M is 1-kb ladder and other lanes represent different saffron genotypes as listed in Table 1.

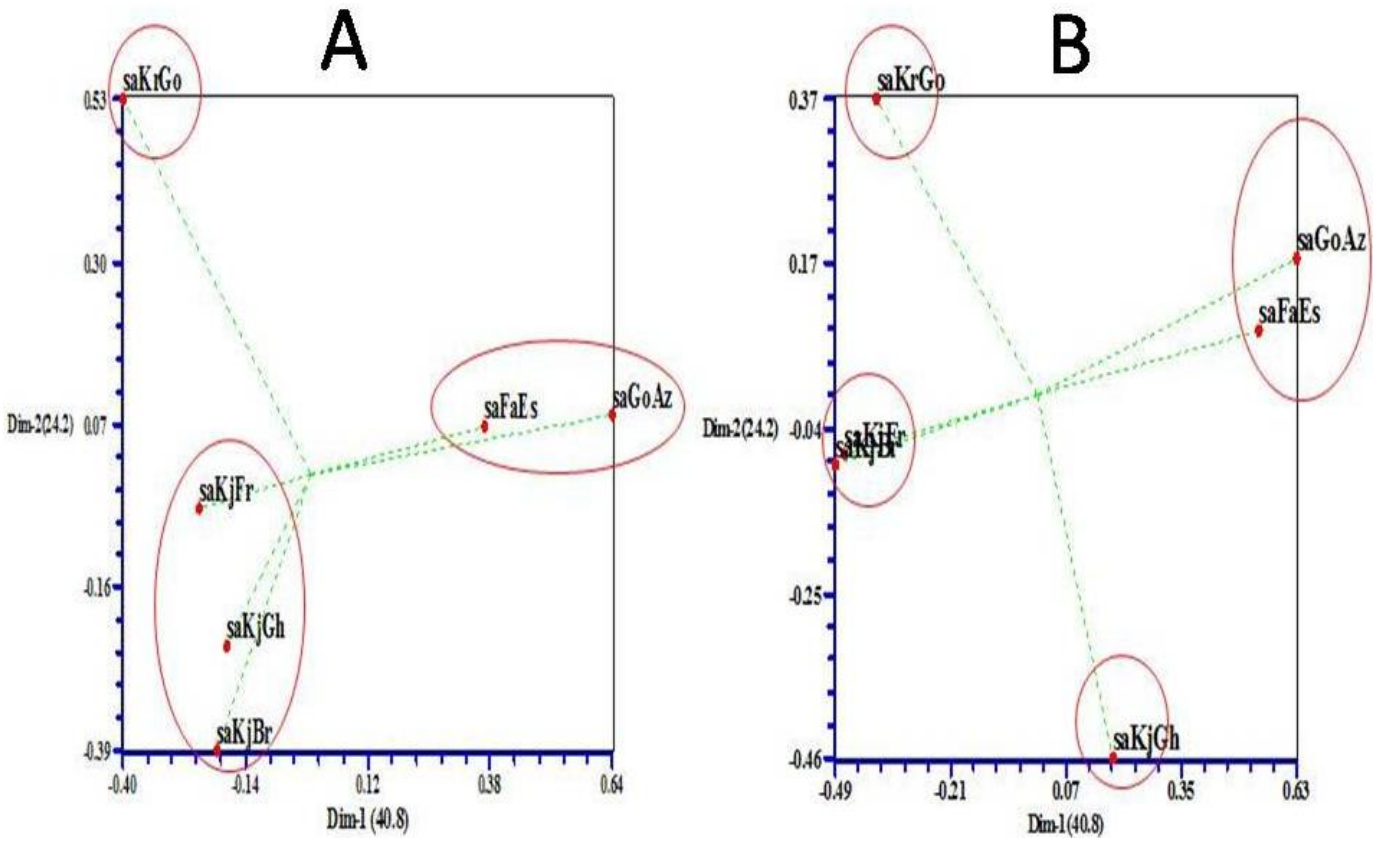

Figure 4. PCoA plots of saffron genotypes (as listed in Table 1) based on RAPD (A) and SRAP (B) data.

from dendrogram and PCoA graphs that the accessions originating from a common place tend to be in the same group.

DISCUSSION

In this study, two different PCR-based approaches:

RAPD and SRAP were carried out for the first time in order to determine the variability of saffron with different geographic origins from Iran. The degrees of polymorphism exhibited by both markers clearly demonstrate their usefulness in genetic analysis of saffron. Accessions were classified into four clusters using both molecular methods, which supplied molecular evidences for the exploit of this rich source in Iran. The cophenetic correlation coefficient (r-value) for RAPD and SRAP dendrogram were 0.83 and 0.97, respectively, suggesting a very good fit between the dendrogram and the corresponding similarity matrices from which they were derived. The existence of differences at the phenotypic level such as size of the flowers, shape of the tepals, differences of colour and intensity in the tepals of samples collected from different origins could be corroborated by molecular analysis, thus confirming the results previously obtained by Beiki et al. (2010), Sik et al. (2008), Estilai (1978), and Gaffari (1986) but not confirming the results obtained by Grilli-Caiola (2004).

Conclusion

SRAP is a novel marker system and preferentially detects polymorphisms in coding sequences, which are more conserved among cultivars and have a relatively low mutation rate. The RAPD marker system, however, detects neutral genetic variation. These markers may have various applications for genetic studies and practical breeding programs in saffron. So, SRAP and RAPD markers were suitable for the molecular characterization and the investigation of phylogenic relationships in saffron. Furthermore, these researches proved that the use of RAPD and SRAP approach were more efficient to examine the genetic diversity in saffron. Though there has been great advancement in the marker technology with the advent of different DNA markers like amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), single nucleotide polymorphism (SNPs) and diversity arrays technology (DArT) still RAPD is quite convenient to apply provided the problem of reproducibility is minimized. The RAPD-PCR products can be cloned and sequenced to convert the RAPD markers in this study into sequence-characterized amplified regions (SCARS), this would increase the detection of co-dominance to improve the utility of the results of RAPD analysis in this study.

The SRAP marker system was a simple and efficient marker system that had several advantages over other systems: simple, with a reasonable throughput rate, able to disclose numerous co-dominant markers, allows easy isolation of bands for sequencing and, most importantly, it targets ORFs, which could be adapted for a variety of purposes in wild relatives including SCAR marker, map construction, and gene chip.

Amplified fragments from SRAP markers could be recovered from the acrylamide gel, re-amplified and sequenced. These sequences could then be compared with other sequences in the databases for annotation. Based on molecular studies, we can identify accessions and species which are suggested for consideration for commercial exploitation and making of hybrids.

RAPD markers were almost as efficient as SRAP markers with regards to detection of polymorphism, number of loci scored and PIC values. Therefore saffron is not monomorphic and we can find diversity for breeding purposes.

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