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GENETIC DIVERSITY OF *CROCUS SATIVUS* AND ITS CLOSE RELATIVE SPECIES ANALYZED BY iPBS-RETROTRANSPOSONS

Aysun GEDIK[1], Duygu ATES[2*], Semih ERDOGMUS[2], Gonul COMERTPAY[3], Muhammed Bahattin TANYOLAC[2**] Hakan OZKAN[1,4**]*

[1]Cukurova University, Department of Biotechnology, Institute of Natural and Applied Sciences, Adana, TURKEY
[2] Ege University, Department of Bioengineering, Izmir, TURKEY
[3] Eastern Mediterranean Agricultural Research Institute, Adana, TURKEY
[4] Cukurova University, Faculty of Agriculture Department of Field Crops, Adana, TURKEY
**: These authors contributed equally to this article*
***Corresponding authors: bahattin.tanyolac@gmail.com, hozkan@cu.edu.tr*

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ABSTRACT

Saffron (*Crocus* L.) is a member of Crocoideae, the biggest of four subfamilies in the Iridaceae family. It has 2n = 3x = 24 chromosomes and is triploid; thus, it is sterile. In previous research, different molecular DNA markers were used but molecular characterization and genetic diversity of this complex genus have not yet been clarified. Therefore, current study aimed to determine the molecular characterization of saffron and its close relative species using inter-primer binding site (iPBS)-retrotransposon markers. Eighty-three iPBS-retrotransposon primers were used in 28 *C. sativus* genotypes and 17 close relative species of saffron to identify their genetic diversity. Sixteen polymorphic iPBS-retrotransposon primers generated a total of 401 polymorphic scorable bands. The mean PIC value, Nei's genetic diversity and Shannon's information index (I) were calculated as 0.85, 0.16 and 0.29, respectively. The results of the Unweighted Pair Group Method with Arithmetic mean UPGMA dendrogram and Principal Coordinates Analysis PCoA analysis indicated a spatial representation of the relative genetic distances among 28 saffron samples and the 17 close relative species were categorized under two distinct groups. Saffron genotypes showed very limited genetic variation and according to the iPBS-retrotransposon data, its close relatives were *C. cartwrightianus* and *C. pallasii* subsp. *pallasii*.

Keywords: *Crocus sativus*, genetic diversity, inter-primer binding site retrotransposons, saffron.

INTRODUCTION

Crocus L. is a member of Crocoideae, the biggest of presently known four subfamilies in the Iridaceae family (Busconi et al., 2015; Goldblatt et al., 2006). While this genus was originally considered to contain approximately 90 small species (Petersen et al., 2008), molecular, morphological and karyological studies undertaken in recent years (Kerndorff et al., 2012; Kerndorff et al., 2013; Harpke et al., 2013; Schneider et al., 2012) have shown that the number of *Crocus* species is presumably higher than 160 (Harpke et al., 2015) and these species are found in a wide range of habitats, including meadows, scrub and woodland (Rubio-Moraga et al., 2009). The majority of *Crocus* species and subspecies are naturally grown in the Balkan Peninsula, Greek, and Turkey (Petersen et al., 2008; Kerndorff and Pasche, 2011; Kerndorff et al., 2012; Peruzzi and Carta, 2011). Among all *Crocus* diversity in the world, Turkey's flora includes a total of 132 *Crocus* species (108 endemic) with different flowering times (Mathew, 1982; Ozhatay, 2002; Kerndorff and Pasche, 2004; Arslan et al., 2007; Alavi-Kia et al., 2008; Yuzbasioglu et al., 2015).

Crocus L. is primarily known for the commercially cultivated species *C. sativus*, generally called "saffron", which is one of the most consumed spices in the world (Negbi, 2003). Saffron and its efficacious components have many applications in many industries such as textile (Mortazavi et al., 2012), perfume (Mousavi and Bathaie, 2011), food (Babaei et al., 2014) and pharmaceuticals (Hadizadeh et al., 2010). In addition, saffron is used as an enhancer of learning ability (Pitsikas and Sakellaridis, 2006), antidepressant (Basti et al., 2007), anti-cancer agent (Abdullaev, 2002; Fernández, 2006), and antioxidant component (Verma and Bordia, 1998). The retail price of saffron can reach 20.000 Euro/kg; thus making it one of the most expensive spices in the world

(Busconi et al., 2015). Due to its great commercial importance, the exploration of the genetic diversity of saffron and relationships between its accessions are significant for germplasm conservation and breeding strategies.

Saffron has 2n=3x=24 chromosomes and is triploid; thus, it is sterile (Gribbon et al., 1999). However, the sterility of this species has limited the application of conventional genetic diversity strategies (Busconi et al., 2015). In the last four decades, classical strategies have been complemented with morphological markers to determine the genetic diversity of saffron; however, DNA markers have the potential to provide more reliable results than morphological markers in genetic diversity research since they represent the variation at the DNA level and are not affected by different environmental conditions (Mondini et al., 2009; Weising et al., 2005; Kumar, 1999; Kumar et al., 2009). Despite this potential, in the literature, there are only few articles reporting on the determination and classification of the systematic and genetic diversity of saffron (Frello and Heslop-Harrison, 2000; Nørbæk et al., 2002; Castillo et al., 2005; Frizzi et al., 2007; Petersen et al., 2008; Seberg and Petersen, 2009; Moraga et al., 2010). These studies have employed different molecular DNA markers such as inter simple sequence repeat (ISSR) (Rubio-Moraga et al., 2009), random amplified polymorphic DNA (RAPD) (Rubio-Moraga et al., 2009), sequence-related amplified polymorphism (SRAP) (Babaei et al., 2014), amplified fragment length polymorphism (AFLP) (Busconi et al., 2015), simple sequence repeats (SSR) (Mir et al., 2015) and inter-retroelement amplified polymorphism (IRAP) (Alsayied et al., 2015; Alavi-Kia et al., 2008) markers but have not fully elucidated the molecular characterization and genetic diversity of this complex genus. Alsayied et al. (2015) reported that according to the IRAP data, *C. sativus* had minimum genetic variation and its ancestors were *C. cartwrightianus* and *C. pallasii* subsp. *pallasii*.

Retrotransposons are mobile genetic elements that generally constitute 50% of plant genome (may reach as high as 90%) and play a significant role in the formation of genetic diversity (SanMiguel et al., 1996; Pearce et al., 1996; Schulman et al., 2012). In addition, retrotransposons are very useful for the development of new markers because of their high prevalence and genome-specific distribution (Schulman et al., 2012). To date, several types of retrotransposons such as retrotransposon-microsatellite and amplified polymorphism (REMAP), IRAP and retrotransposon-based insertion polymorphism (RBIP) have been used for research on plant characterization (Kalendar et al., 2011; Schulman et al., 2012; Alavi-Kia et al., 2008; Alsayied et al., 2015). However, the process of developing retrotransposon markers for a new plant species has certain restricting factors such as the variation of the polymerase chain reaction (PCR) product size and the necessity of cloning and sequence information to design primers that match the flanking genomic DNA at each specific site (Kalendar et al., 2010). Recently, to reduce the negative effect of these factors, Kalendar et al. (2010) proposed a new highly effective and universal molecular marker system based on the conversed sequences of retrotransposons, called "inter-primer binding site (iPBS) retrotransposons". In particular, the amplification technique of the iPBS-retrotransposons is very practical and comprises a powerful DNA fingerprinting technology that does not require prior sequence information. Thus, reproducibility is very high owing to the primer length and strong stringency success by selecting appropriate annealing temperature (Guo et al., 2014). The iPBS-retrotransposon marker system has been successfully implemented in phylogenetic studies, clone identification research, and genetic diversity analyses (Smýkal et al., 2011; Baránek et al., 2012; Gailite and Rungis, 2012). To date, genetic diversity evaluation of certain plant species such as flax (Smýkal et al., 2011), apricot (Baránek et al., 2012), *Saussurea* (Gailite and Rungis, 2012), chickpea (Andeden et al., 2013), rice (Comertpay et al., 2015), lentil (Baloch et al., 2015b) and pea (Baloch et al., 2015a) have been studied using iPBS-retrotransposons; however, to the best of our knowledge, the current study is the first to determine the molecular characterization of saffron and its close relative species using this marker system.

MATERIALS AND METHODS

Plant materials and DNA extraction

In the current study, a total of 28 *C. sativus* samples collected in Turkey, Iran, Spain, Azerbaijan, Italy, France and Greek and 17 close relative species of saffron collected in Turkey and Greek were used as plant material. Table 1 presents the number of genotypes, taxon and collection site of each *C. sativus* sample and Table 2 gives information on the close relative species of these samples.

Table 1. *List of* C. sativus *samples used for molecular characterization analysis.*

Genotype number	Genotype name	Taxon	Collection site
1	Tokat 1	*C. sativus*	Tokat / Turkey
2	Tokat 2	*C. sativus*	Tokat / Turkey
3	Tokat 3	*C. sativus*	Tokat / Turkey
4	Tokat 4	*C. sativus*	Tokat / Turkey
5	Tokat 5	*C. sativus*	Tokat / Turkey
6	Iranian 1	*C. sativus*	Iranian
7	Iranian 2	*C. sativus*	Iranian
8	Iranian 3	*C. sativus*	Iranian
9	Iranian 4	*C. sativus*	Iranian
10	Iranian 5	*C. sativus*	Iranian
11	AHBR	*C. sativus*	Kastamonu / Turkey
12	49	*C. sativus*	Kastamonu / Turkey
13	50	*C. sativus*	Kastamonu / Turkey
14	51	*C. sativus*	Kastamonu / Turkey
15	52	*C. sativus*	Kastamonu / Turkey
16	53	*C. sativus*	Kastamonu / Turkey
17	54	*C. sativus*	Kastamonu / Turkey
18	61	*C. sativus*	Kastamonu / Turkey
19	62	*C. sativus*	Kastamonu / Turkey
20	AABB	*C. sativus*	Kastamonu / Turkey
21	BCV 001584	*C. sativus*	Spain
22	BCV 001687	*C. sativus*	Iranian
23	BCV 001783	*C. sativus*	Azerbaijan
24	BCV 001806	*C. sativus*	Italy
25	BCV 002708	*C. sativus*	France
26	BCV 002911	*C. sativus*	Greek
27	5	*C. sativus*	Kastamonu / Turkey
28	SC77	*C. sativus*	Kastamonu / Turkey

Table 2. *List of close relative species of saffron samples used for molecular characterization analysis.*

Genotype number	Genotype name	Taxon	Collection site
29	SC2	*C. pallasii subsp. pallasii*	Mugla / Turkey
30	SC5	*C. pallasii* subsp. *pallasii*	Mugla / Turkey
31	SC1	*C. pallasii subsp. pallasii*	Mugla / Turkey
32	SC8	*C. pallasii* subsp. *pallasii*	Mugla / Turkey
33	SC10	*C. pallasii* subsp. *pallasii*	Izmir / Turkey
34	SC12	*C. pallasii* subsp. *pallasii*	Denizli / Turkey
35	SC17	*C. pallasii* subsp. *pallasii*	Konya / Turkey
36	SC18	*C. pallasii* subsp. *pallasii*	Konya / Turkey
37	BZDG	*C. pallasii* subsp. *pallasii*	Izmir / Turkey
38	DDGL	*C. pallasii* subsp. *pallasii*	Isparta / Turkey
39	SB60	*C. pallasii* x *C. mathewi*	Antalya / Turkey
40	SC9	*C. mathewii*	Antalya / Turkey
41	SC20	*C. pallasii* subsp. *dispathaceus*	Mersin / Turkey
42	SC21	*C. pallasii* subsp. *dispathaceus*	Mersin / Turkey
43	SC22	*C. pallasii* subsp. *turcicus*	Gaziantep / Turkey
44	SC45	*C. asumaniae*	Antalya / Turkey
45	CRTW	*C. cartwrightianus*	Greek

The total genomic DNA was extracted from all samples using the method described by Doyle and Doyle (1987), with some minor modifications (Kafkas, 2006). Then, the purity of the genomic DNA was visually checked on agarose gel (1%) and total DNA concentration was measured using a Qubit®2.0 Fluorometer (Life Technologies, US). The final DNA concentration was adjusted to 5 ng/μL to use in PCR.

Analysis of iPBS-retrotransposon markers

In current study, 83 iPBS-retrotransposon primers developed by Kalendar et al. (2010) were used. DNA

amplification was performed according to the protocol of Andeden et al. (2013). PCR amplifications were carried out using 25µl reaction mixture containing 5 ng template DNA, 3 x Dream *Taq* Green PCR buffer (Fermentas), 10 µM of primer (single primer), 5 mM dNTPs, and 1.75-unit Dream *Taq* DNA polymerase (Fermentas).

The thermal cycling profile of the PCR was as follows: initial denaturation at 95 ˚C for 4 min, 30 cycles of 95 ˚C for 15 s, 50-65 ˚C for 1 min and 68 ˚C for 1 min; and final extension at 72 ˚C for 5 min (Kalendar et al., 2010). For the separation of all the PCR products, 1.7-2 % (w/v) agarose gel electrophoresis was used with 1 x TBE buffer for 2 h, stained with ethidium bromide and visualized by a G-BOX gel documentation system (Syngene, USA).

Scoring and analysis of the data

The DNA bands of each iPBS-retrotransposon primer were manually scored. Only the clear and strong bands were considered and the faint bands were ignored to exclude artificial bands. To build binary matrices, the presence or absence of an iPBS-retrotransposon band at a particular locus was scored as "1" and "0", respectively. The polymorphism information content (PIC) for dominant iPBS-retrotransposon markers was calculated by the formula described by de Riek et al. (2001) and Baloch et al. (2015a);

$$PIC_i = 2f_i (1 - f_i)$$

where "f_i" is the band present (amplified allele) frequency of the marker and "$(1 - f_i)$" refers to the band absent (null allele) frequency of the marker. The NTSYSpc 2.11 V software package (Rohlf, 2000) was used for the analysis of the iPBS binary matrices. Dendrograms were constructed based on Dice's genetic similarity coefficient (Nei and Li, 1979) using the unweighted pair-group method with arithmetic averages (UPGMA). To calculate Nei's distance coefficient (h), the Splits Tree4 software package (Huson and Bryant, 2006) was used on the binary data and then a Neighbor-Net tree (Nei, 1987) was drawn. Shannon's information index (I) was calculated using the R computer program.

RESULTS

iPBS-retrotransposon marker system

Eighty-three iPBS-Retrotransposon primers were tested on 4 saffron genotypes for initial screening. Of these, 16 primers represented (19%) a large number of good and clear band profiles. Therefore, these 16 primers yielding reproducible polymorphic bands were selected for the amplifications of all 28 saffron samples and its 17 close relative species. The 16 iPBS-retrotransposon primers generated a total of 401 polymorphic scorable bands (Table 3). The number of polymorphic bands per primer ranged from 15 (iPBS 2238) to 35 (iPBS 2081), with an average of 25 bands per primer (Table 3).

Table 3. *Information about the iPBS-Retrotransposon primers used in current study.*

Primer Number	Primer Name	NTB	NSPB	NSPB %	PIC	h	I
1	iPBS 2075	25	25	6.23	0.85	0.20	0.36
2	iPBS 2079	36	34	8.48	0.86	0.12	0.22
3	iPBS 2081	35	35	8.73	0.89	0.15	0.30
4	iPBS 2085	21	19	4.74	0.82	0.19	0.31
5	iPBS 2095	26	24	5.98	0.73	0.23	0.33
6	iPBS 2219	22	22	5.49	0.90	0.12	0.25
7	iPBS 2229	28	28	6.98	0.87	0.14	0.28
8	iPBS 2238	15	15	3.74	0.79	0.17	0.25
9	iPBS 2239	26	26	6.48	0.82	0.20	0.34
10	iPBS 2273	21	20	4.99	0.92	0.10	0.21
11	iPBS 2375	23	23	5.73	0.84	0.16	0.26
12	iPBS 2376	25	25	6.23	0.88	0.15	0.28
13	iPBS 2381	31	30	7.48	0.82	0.18	0.28
14	iPBS 2400	26	26	6.48	0.88	0.12	0.23
15	iPBS 2401	30	28	6.98	0.84	0.18	0.31
16	iPBS 2415	23	21	5.24	0.83	0.19	0.37
Total	-	413	401	-	-	-	-
Average	-	-	-	-	0.85	0.16	0.29

NTB; number of total bands. NSPB; number of scorable polymorphic bands.
PIC; polymorphism information index. h; Nei's gene diversity. I; Shannon's information index.

The PIC value was used to calculate the effectiveness of polymorphic loci in defining genetic diversity among all genotypes. The mean PIC value was determined as 0.85, ranging from 0.73 (iPBS 2095) to 0.92 (iPBS 2273) (Table 3) indicating that the iPBS-retrotransposon primers had the potential to develop high loci polymorphism.

Genetic diversity between saffron and its close relative species

The data on each iPBS-retrotransposon was interpreted using the following statistical parameters; PIC, Nei's genetic diversity (h), and Shannon's information index (I). These parameters were evaluated for all the genotypes as a single population. Nei's genetic diversity among saffron genotypes and its close relative species varied from 0.10 (iPBS 2273) to 0.23 (iPBS 2095), with a mean value of 0.16 and Shannon's information index (I) varied from 0.21 (iPBS 2273) to 0.37 (iPBS 2415), with a mean value of 0.29 (Table 3).

The iPBS-retrotransposon analysis predicted that the highest genetic variation was between CRTW and SC9 while the lowest genetic variation was between BCV 001687, BCV 001584, BCV 001783, BCV 001806, and BCV 002911.

Cluster and principal component analysis for the iPBS-retrotransposon markers

According to the results of the unweighted pair group analysis with the arithmetic mean (UPGMA) dendrogram including a heat map, 28 saffron samples and its 17 close relative species were under two main clusters (Figures 1 and 2). The first cluster contained 28 saffron genotypes as listed in Table 1 and the second cluster comprised 17 close relative species as shown in Table 2. This indicates that there was a good differentiation between the saffron genotypes and its close relative species. The two main groups were further divided into different subgroups with very low variability.

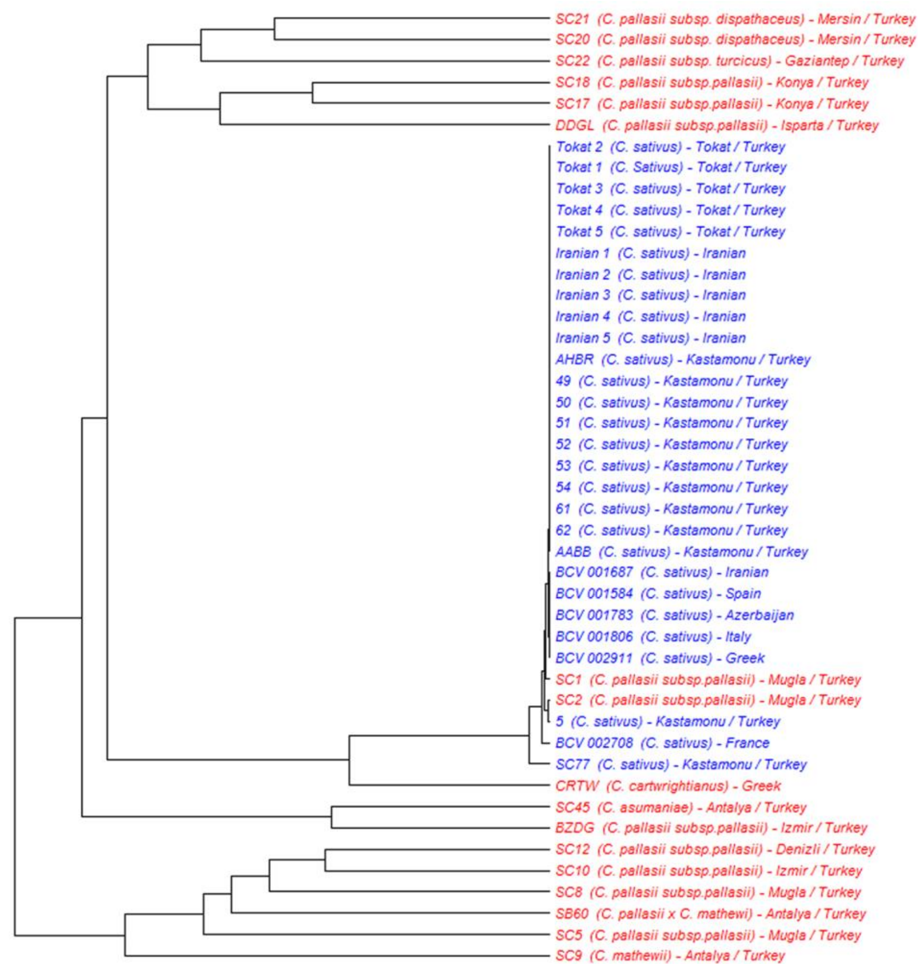

Figure 1. UPGMA dendrogram of 45 saffron genotypes and its close relative species based on iPBS-retrotransposon primers.

The results of the principal component analysis indicated two distinct groups in the spatial representation of the relative genetic distances among 28 saffron samples and its 17 close relative species (Figure 3), supporting the results presented in the UPGMA dendrogram (Figures 1 and 2).

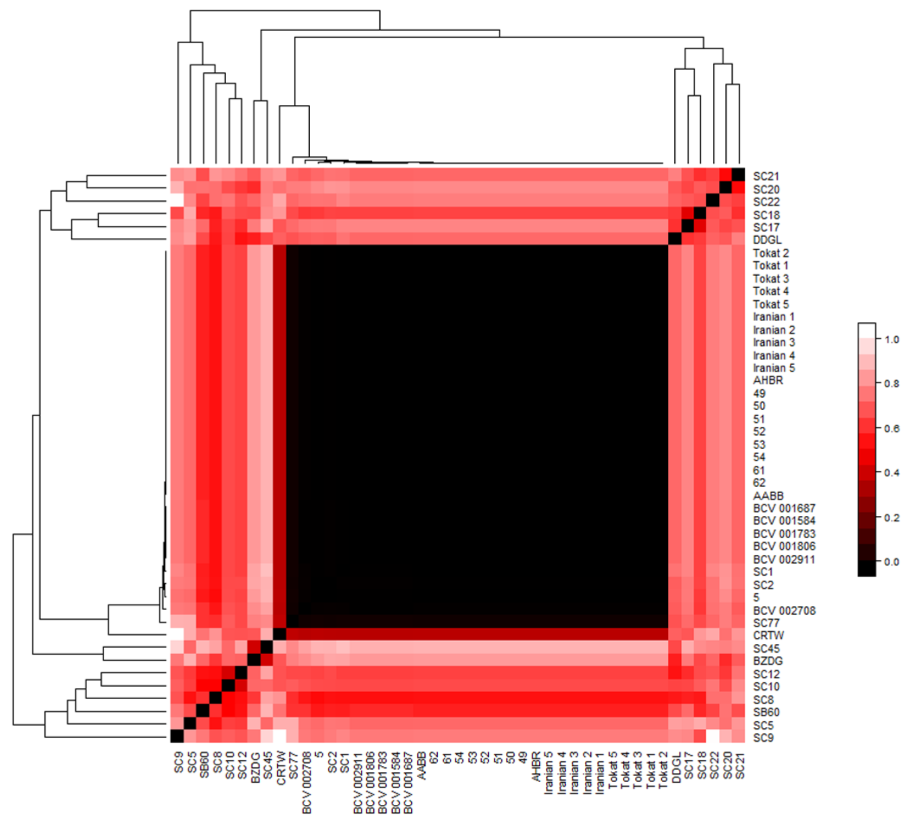

Figure 2. The heat map obtained from the analysis of iPBS-retrotransposon data.

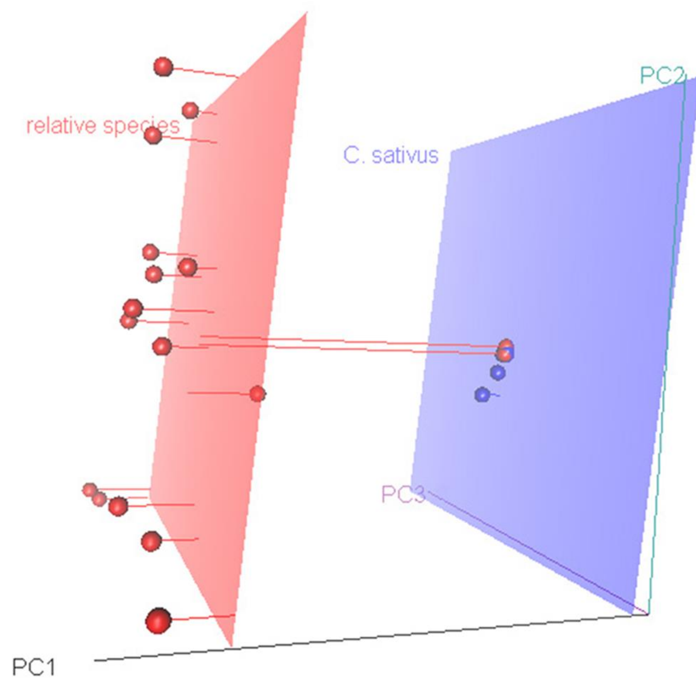

Figure 3. The relationships between the 45 saffron genotypes and its close relative species visualized by PCoA using the iPBS-retrotransposon markers.

DISCUSSION

Molecular genetic markers are advantageous for defining genetic diversity in plant breeding. Transposable elements, especially the retrotransposons, include overmuch plant genomes and replication of retrotransposons generates genetic diversity, an ability that makes them a brilliant source of molecular genetic markers (Schulman et al., 2004). These markers have been widely used in numerous studies on the genetic diversity of different plant species such as bread wheat (Gribbon et al., 1999), barley (Manninen et al., 2000), tomato (Tam et al., 2005), rice (Branco et al., 2007), *Aegilops tauschii* (Saeidi et al., 2008), *Crocus* (Alavi-Kia et al., 2008), triticale (Bento et al., 2008), *Citrus* and its relatives (Biswas et al., 2010), and grapevine (D'Onofrio et al., 2010). However, development of retrotransposon markers for a new plant species has certain restricting factors (Kalendar et al., 2010). Recently, Kalendar et al. (2010) proposed the iPBS-retrotransposons marker technique as a universal molecular marker based on the principle of using a single primer amplified region as a forward and reverse (Mehmood et al., 2013). These markers have been reported to amplify effectively for genetic diversity analyses (Baránek et al., 2012; Andeden et al., 2013) since the primers used for PCR can anneal to genomes that include diverse LTR sequences (Kalendar et al., 2010). However, despite being universal, to date, iPBS-retrotransposons markers have only been used in the genetic diversity research of only few plants and it has not been employed to the characterization of saffron genotypes and its wild relatives.

Analysis of iPBS-retrotransposon markers

In current study, a total of 83 iPBS-retrotransposon primers were screened and 16 (19.3 %) (12-18 nt long) presented a total of 401 good, clear and polymorphic band profiles (Table 3). Similarly, Mehmood et al. (2013), Guo et al. (2014) and Nemli et al. (2015) screened 83, 41 and 83 iPBS-retrotransposon primers, respectively and selected 6, 15 and 47 primers, respectively for further analysis. These results indicate that these iPBS-retrotransposon primers are not conserved among saffron, common bean, grape, guava, and other species investigated by Kalendar et al. (2010).

The average number of polymorphic iPBS-retrotransposon bands found in the current study (25.1 bands) was far greater than those reported by Baránek et al. (2012) (7.1 bands), Andeden et al. (2013) (13 bands), Mehmood et al. (2013) (16.2 bands), Fang-Yong and Ji-Hong (2014) (8.3 bands), Guo et al. (2014) (6.6 bands), and Nemli et al. (2015) (3.8 bands). This could be due to saffron having a larger genome size compared to the plant genomes studied previously. These results may also indicate that iPBS-retrotransposon primers for saffron are more conserved compared to apricot (Baránek et al., 2012), C*icer* species (Andeden et al., 2013), guava (Mehmood et al., 2013), *Myrica rubra* (Fang-Yong and Ji-Hong, 2014), grape (Guo et al., 2014), and common bean (Nemli et al., 2015). On the other hand, genetic diversity studies on saffron have emphasized that iPBS-retrotransposon primers are more informative than other methods such as AFLP (Zubor et al., 2003), RAPD (Rubio-Moraga et al., 2009; Mir et al., 2015), SSR (Rubio-Moraga et al., 2009; Mir et al., 2015), ISSR (Rubio-Moraga et al., 2009; Mir et al., 2015), and SRAP (Babaei et al., 2014). In these studies, the iPBS-retrotransposon primers generated numerous bands with a high number of copies in the plant genomes investigated. The amplified band sizes of different iPBS-Retrotransposon primers used in the current study were within the range of 100-2000 bp, consistent with the results reported by Mehmood et al. (2013), Fang-Yong and Ji-Hong (2014), and Nemli et al. (2015).

The PIC value was utilized to calculate the efficiency of polymorphic loci in identifying genetic diversity (Babaei et al., 2014) and define the discriminating power of marker among genotypes (Nemli et al., 2015). In the current study, the mean PIC value was calculated as 0.85, ranging from 0.73 (iPBS 2095) to 0.92 (iPBS 2273) (Table 3). This result indicates that iPBS 2273 was the most efficient and informative primer for discriminating among saffron genotypes. The PIC values detected in the present study were higher than those reported in previous saffron studies using other genetic marker systems. For example, Babaei et al. (2014) used SRAP markers to detect genetic diversity among saffron samples and reported the mean PIC value to be 0.15. Mir et al. (2015) used RAPD, SSR and ISSR markers to determine the molecular characterization of saffron and found the average PIC values to be 0.03 and 0.018 for RAPD and ISSR markers, respectively. On the other hand, in that study, SSR markers did not show any polymorphism. Similarly, Rubio-Moraga et al. (2009) reported that ISSR markers did not show any polymorphism in saffron. The high PIC value in the current study may be due to the large variation in the number of loci observed for iPBS-Retrotransposon markers compared to AFLP, SRAP, RAPD, SSR and ISSR markers (Babaei et al., 2014; Rubio-Moraga et al., 2009; Mir et al., 2015; Zubor et al., 2003).

Since the *C. sativus* genotypes undergoes vegetative proliferation (Babaei et al., 2014), other molecular genetic markers are not able to detect or identify very low polymorphism among saffron genotypes (Babaei et al., 2014; Mir et al., 2015; Rubio-Moraga et al., 2009), iPBS-retrotransposon markers are a very effective technique for the assessment of genetic diversity among saffron genotypes as confirmed by the high PIC values obtained in current study.

Genetic diversity between saffron and its close relative species

In current study, the average Nei's genetic diversity (h) and Shannon's information index (I) among saffron genotypes and its close relative species were 0.16 and 0.29, respectively (Table 3), indicating a low level of differentiation. Similarly, the mean Shannon's information index for iPBS-retrotransposon markers was reported to

be 0.12 (Yildiz et al., 2015) and 0.27 by (Mehmood et al., 2013).

The results of iPBS-retrotransposon analysis showed that the highest genetic variation was between CRTW (*C. cartwrightianus*) and SC9 (*C. mathewii*). Alsayied et al. (2015) reported that *C. cartwrightianus* and *C. mathewii* were genetically distant from each other. In current study, all the *C. sativus* genotypes were in the same cluster with minimum genetic variation, but they tended to group based on their geographical origin. This showed that genotypes from the same location have no genetic variation but small genetic variation may be observed among localities (Caiola et al., 2004).

Cluster and principal component analysis for the iPBS-retrotransposon markers

The genetic diversity among saffron genotypes and its close relative species was clearly presented in the UPGMA dendrogram constructed from the DNA profile. This dendrogram placed 28 saffron genotypes and its 17 close relative species under two main clusters closely related with highly low diversity (Figures 1 and 2). The results of the principal component analysis also indicated the presence of two distinct groups based on the spatial representation of the relative genetic distances among 28 saffron samples and its 17 close relative species (Figure 3), supporting the results from the UPGMA dendrogram in that saffron genotypes had very limited genetic variation. This can be explained by the high degree of commonness in these genotypes due to the sterile and triploid nature of saffron species that vegetatively propagate by their corm, not undergoing sexual reproduction (Mir et al., 2015; Fernández, 2004).

In the UPGMA dendrogram, all the *C. sativus* genotypes (n = 28), *C. cartwrightianus* and some *C. pallasii* subsp. *pallasii* were included in the same group (Figures 1-3). *C. cartwrightianus* has morphological similarity to *C. sativus* and studies that performed a karyotype and morphology analysis on the genotypes allied to *C. sativus* demonstrated that *C. cartwrightianus* is one of the ancestors of *C. sativus* (Alsayied et al., 2015; Caiola et al., 2004; Larsen et al., 2015) (Alsayied et al., 2015). Similar to current results, Alsayied et al. (2015) concluded, based on the IRAP data, that *C. sativus* had minimum genetic variation and its ancestors were *C. cartwrightianus* and *C. pallasii* subsp. *pallasii*. These findings are also supported by the flow sorting analysis of *C. sativus* and its close relatives (Brandizzi and Grilli Caiola, 1998; Erol et al., 2014). AFLP and RAPD fingerprinting also revealed *C. cartwrightianus* to be the closest relative of *C. sativus* (Zubor et al., 2003; Caiola et al., 2004). In addition, the flow cytometric analysis of *Crocus* genotypes suggested that *C. cartwrightianus* was the most likely ancestor of *C. sativus* (Brandizzi and Grilli Caiola, 1998). In the current study, *C. pallasii* subsp. *pallasii*, *C. asumanie* and *C. mathewii* were in the second group in the UPGMA dendrogram (Figures 1-3). Although the entire *C. sativus* iPBS-retrotransposon profile was different from that of all the genotypes analyzed in current study, it was most similar to that of *C. pallasii* subsp. *pallasii*, *C. asumanie,* and *C. mathewii* (Figures 1-3). Erol et al. (2014) supported these findings in their flow sorting study.

The general consensus regarding a good genetic molecular marker system is that it should be polymorphic, cheap, quick, and equally distributed across the whole genome. The marker should be easy to use, be able to differentiate between genetic differences, and require a low amount of DNA and no previous knowledge of the genome (Odong et al., 2011). The findings from the present study confirm that iPBS-retrotransposon markers satisfy all these criteria for both *Crocus sativus* and its close relative.

CONCLUSION

iPBS-retrotransposon markers are less powerful for detection of genetic diversity in close relative species according to the average Nei's genetic diversity (h) and Shannon's information index (I).

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