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DIVERSITY AND PHYLOGENY OF SAFFRON (*Crocus sativus* L.) ACCESSIONS BASED ON IPBS MARKERS

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This study sought to measure genetic diversity and phylogenetic structure among 196 individuals of saffron from 20 geographically separate accessions from Iran, Spain and Turkey using retrotransposon derived iPBS markers. Twenty-eight primers amplified a total of 179 polymorphic alleles with an average of 6.4 bands per primer. The average of parameters shannon's information index, genetic distance and gene diversity was 0.483, 0.286 and 0.841 respectively. Polymorphic information index ranged from 0.407 to 0.953 with an average of 0.824. Primers 2298, 2229 and 2393 with 0.953, 0.943 and 0.943 PIC respectively, identified as the most informative primers in this study. The results of phylogenetic trees showed that twenty saffron accessions were placed into four major clusters that matched with their geographical locations completely. These results are supported by principal coordinate analysis. Overall, we can confirm that iPBS markers as low cost and high efficient molecular markers are a powerful DNA fingerprinting for assessing genetic diversity and phylogenetic analysis among saffron accessions originating from different geographical regions.

Key words: Cluster analysis, diversity, inter-primer binding site (iPBS), molecular markers, retrotransposon, saffron (*Crocus sativus* L.).

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INTRODUCTION

Nowadays, saffron (*Crocus sativus* L.) is a plant with numerous applications in medicine and food industries, known as the most expensive spice and the commercial plant in the world (FERNÁNDEZ, 2006; MORAGA *et al.*,, 2009). *C. sativus* is classified in the family *Iridaceae* (*Iris*) in the genus Crocus, which consists of about probably 200 species or even more (MATHEW, 1982). It is male infertile species, therefore, proliferated vegetatively by corms (FERNÁNDEZ and PANDALAI, 2004; GRESTA *et al.*,, 2009). The range of saffron cultivation in the world shows that a wide range of adaptability to soil types, temperatures and day length, encourage its production from Mediterranean basin to middle east (KAFI, 2006; MORAGA *et al.*,, 2009). At present, Iran which produces over 93.7% of its global supply, is known as the largest saffron producer in the world (GHORBANI, 2007).

Investigations of genetic diversity and population structure can provide important information for the management of genetic resources and the conservation of biodiversity in plants (ODONG *et al.*,, 2011). Nowadays, molecular markers have been utilized to differentiate, identify and evaluate genetic variation of many cultivars (KARIM *et al.*,, 2010). Because these markers are not affected by plant developmental processes or environmental influences, conclusions and interpretations could be more valuable and reliable than morphological markers (KUMAR *et al.*,, 2009; MEHMOOD *et al.*,, 2013).

Retrotransposons are a type of transposable element consisting of mobile DNA sequences within eukaryotic genomes (WESSLER, 2006). In higher plants, they can constitute more than half of the repetitive DNA (SCHNABLE *et al.*,, 2009), and are dynamic genome components with the ability to integrate new copies and facilitate intra-chromosomal recombination (BELYAYEV *et al.*,, 2010; MONDEN *et al.*,, 2014). Several PCR-based marker methods have been developed to reveal insertional polymorphisms of long terminal repeat (LTR) retrotransposons, including retrotransposon-based insertion polymorphism (RBIP), interretrotransposon amplified polymorphism (IRAP), retrotransposon-microsatellite and amplified polymorphism (REMAP) (KALENDAR *et al.*,, 2011; SCHULMAN *et al.*,, 2012). Recently, another exceedingly universal and efficient molecular marker based on the conversed sequences of retrotransposons, i.e., inter-primer binding site (iPBS), has been developed by KALENDAR *et al.*, (2010). The iPBS method has several advantages for screening diverse LTR sequences and conducting DNA fingerprinting without the need for prior sequence knowledge. Additionally, it is a fast, low-cost and efficient molecular method applicable to plant breeding (MEHMOOD *et al.*,, 2013). The retrotransposon-based markers were successfully applied in studying the genetic diversity and relationships in plants (ANDEDEN *et al.*,, 2013; BARÁNEK *et al.*,, 2012; GAILĪTE *et al.*,, 2011; GAILITE and RUNGIS, 2012; GUO *et al.*,, 2014; MEHMOOD *et al.*,, 2013; SMÝKAL *et al.*,, 2011).

Until now, numerous researchers evaluated variation of saffron accessions at phenotypic and genotypic levels. It has been reported that there were phenotypic variations within cultivated *C. sativus* (CAIOLA *et al.*,, 2000; WEISING *et al.*,, 2005). But there are contradictory results about genetic diversity of saffron, some researchers showed that saffron is a monomorphic plant (ALAVI-KIA *et al.*,, 2008; CAIOLA *et al.*,, 2004; MORAGA *et al.*,, 2009). On the other hand, some researchers detected some polymorphisms among saffron accessions by using ISSR (SIK *et al.*,, 2008), RAPD and SRAP (KEIFY and BEIKI, 2012), SSR (NAMAYANDEH *et al.*,, 2013; NEMATI *et al.*,, 2012), SRAP (BABAEI *et al.*,, 2014), RAPD and AFLP (EROL *et al.*,, 2014).

Due to the importance of saffron as a commercial plant, evaluation of its genetic diversity among accessions is critically important for breeding and germplasm conservation. At present,

iPBS marker has not been identified in saffron. Hence, this study was conducted to assess the ability of iPBS markers for differentiating within the saffron accessions collected from different locations in Iran and to compare these accessions with accessions originating from Spain and Turkey.

MATERIALS AND METHODS

Plant materials

In this study, twenty saffron accessions collected from the most ancient cultivated locations in Iran as well as from Spain and Turkey were analyzed. Ten individuals of each accession of Iran (18 accessions) and ten individuals of 1 accession from Turkey and six individuals of 1 accession from Spain (a total of 196 individuals) were tested in the plant biotechnology laboratory of EBILTEM Institute, EGE University, Turkey. The details of the accessions and their geographic origins are listed in Table 1.

Table 1. Locations, altitudes of Crocus species collected from different regions of Iran, Spain and Turkey

Population no.	Regional origin	Country	Altitude (M)	Latitude (N)	Longitude (E)	No. individual
1	Roshtkhar	Iran	1140	59° 37′	34° 58′	10
2	Yazd	Iran	1230	54° 37′	31° 89′	10
3	Kerman	Iran	2250	57° 79′	30° 07′	10
4	Birjand	Iran	1491	59° 12′	32° 52′	10
5	Bardaskan	Iran	985	57° 57′	35° 15′	10
6	Esfehan	Iran	1570	51° 65′	32° 63′	10
7	Fars	Iran	1486	52° 32′	29° 37′	10
8	Gonabad	Iran	1450.8	59° 13′	35° 16′	10
9	Ferdos	Iran	1293	58° 72′	34° 18′	10
10	Bejestan	Iran	1265	58° 31′	34° 94′	10
11	Torbat heydarieh	Iran	950.4	35° 60′	15° 35′	10
12	Zaveh	Iran	2924	59° 18′	35° 28′	10
13	Ghaen	Iran	1432	59° 10′	33° 43′	10
14	Kashmar	Iran	1063	58° 19′	35° 64′	10
15	Torbat jam	Iran	1056	58° 41′	34° 21′	10
16	Neyshaboor	Iran	1250	58° 19′	35° 40′	10
17	Gorgan	Iran	155	54° 48′	36° 83′	10
18	Mashhad	Iran	999.2	59° 38′	36° 16′	10
19	Izmir	Turkey	7	27° 08′	38° 29′	10
20	Barcelona	Spain	12	2° 18′	41° 38′	6

DNA extraction

DNA was extracted from saffron corm (30 to 50 mg) of each individual according to the method of Beiki et al. (2011). DNA concentration was estimated by a spectrophotometer and in a 2.0% agarose gel stained with ethidium bromide. All isolated DNA samples were diluted to 5 ng/µl and stored at −20°C for iPBS method.

iPBS PCR amplification

This study presented the first primary set of iPBS markers for evaluating genetic relationships among saffron accessions (*C. sativus* L.). Initially, 30 iPBS primers designed by KALENDAR *et al.*, (2010) were tested on some DNA samples and 28 primers were selected with

high clarity and repeatability for polymorphic assessment in studied saffron accessions (Table 2). DNA amplification was carried out by using a modified protocol of KALENDAR *et al.,* (2010). The PCR was performed in a 15 µl reaction mixture containing 6 µl DNA (30 ng), 1.5 mM of 10X PCR buffer, 1 µM of primer, 1.2 mM dNTPs, 0.2 unit Taq DNA polymerase (GoTaq, Promega), 2 mM $MgCl_2$. The PCR program had an initial hot start at 94°C for 3 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 43-60°C for 30 seconds and extension at 72°C for 2 minute. Following this was a final extension at 72°C for 5 minutes and then the program was terminated by holding at 4°C. Amplification products were separated by electrophoresis on 3% agarose gel in 1X TBE buffer and detected by staining with ethidium bromide. Ten microliters of amplified DNA were applied in each well of the gel. One kb ladder was used to estimate fragment lengths. The gels were run at a current of 50 mA until the bromophenol had migrated 10cm from the well. The bands were then visualized under UV light and photographed.

Table 2. Features of twenty eight iPBS primers used to amplify twenty genomic DNA templates of saffron accessions and the amplified DNA products

Primer	Sequence (5′→3′)	Annealing temperature (◦C)	No. of polymorphic bands	Shannon's Information Index (I)	Gene diversity (h)	Polymorphic information index (PIC)
2080	CAGACGGCGCCA	47	8	0.343	0.641	0.618
2226	CGGTGACCTTTGATACCA	50	10	0.362	0.794	0.783
2229	CGACCTGTTCTGATACCA	48	10	0.582	0.945	0.943
2232	AGAGAGGCTCGGATACCA	43	4	0.512	0.863	0.848
2237	CCCCTACCTGGCGTGCCA	65	6	0.597	0.924	0.920
2244	GGAAGGCTCTGATTACCA	60	8	0.481	0.892	0.886
2249	AACCGACCTCTGATACCA	47	6	0.598	0.895	0.887
2252	TCATGGCTCATGATACCA	45	7	0.425	0.844	0.827
2273	GCTCATCATGCCA	45	8	0.345	0.856	0.842
2277	GGCGATGATACCA	47	8	0.483	0.914	0.909
2298	AGAAGAGCTCTGATACCA	45	8	0.597	0.955	0.953
2389	ACATCCTTCCCA	48	3	0.389	0.486	0.407
2087	GCAATGGAACCA	48	5	0.380	0.819	0.795
2380	CAACCTGATCCA	50	7	0.547	0.879	0.873
2391	ATCTGTCAGCCA	45	5	0.519	0.883	0.874
2393	TACGGTACGCCA	43	8	0.521	0.937	0.934
2394	GAGCCTAGGCCA	57	5	0.458	0.846	0.829
2395	TCCCCAGCGGAGTCGCCA	60	3	0.499	0.607	0.530
2415	CATCGTAGGTGGGCGCCA	47	6	0.344	0.761	0.728
2085	ATGCCGATACCA	55	7	0.455	0.898	0.893
2272	GGCTCAGATGCCA	53	7	0.415	0.921	0.916
2074	GCTCTGATACCA	53	6	0.551	0.847	0.829
2075	CTCATGATGCCA	53	4	0.517	0.798	0.777
2222	ACTTGGATGCCGATACCA	47	7	0.576	0.880	0.871
2270	ACCTGGCGTGCCA	47	6	0.590	0.860	0.849
2381	GTCCATCTTCCA	55	5	0.550	0.867	0.853
2382	TGTTGGCTTCCA	55	8	0.387	0.900	0.892
2401	AGTTAAGCTTTGATACCA	53	4	0.513	0.688	0.630
Total	---	---	179	13.536	23.397	22.896
Average	---	---	6.39	0.483	0.841	0.824

Data scoring and analysis

The amplified bands were manually scored as either 1 (present) or 0 (absent). Only clear, repetitive and well-separated bands were selected for scoring. Number of alleles per locus, Nei's gene diversity (h), polymorphism information content (PIC), genetic distance (GD) and Shannon's information index (I) were calculated using Power Marker ver. 3.25 (LIU and MUSE, 2005). Polymorphism information content (PIC) was calculated for each marker using equation 1, according to the ANDERSON *et al.*, (1993) method.

$$PIC_j = 1 - \sum_{i=1}^{n} p_i^2 \qquad \text{(Eq. 1)}$$

Where, i= i^{th} allele of j^{th} marker, n= number of alleles at j^{th} marker and p= allele frequency. The PIC value can range from 0 to 1. At a PIC of 1, the marker would have a unlimited number of alleles. At a PIC of 0, the marker has only one allele. PIC value explains diversity within accessions (intra-population diversity) and determines the degree of polymorphism in each locus, a PIC value of less than 0.25 showing low polymorphism, a value between 0.25 and 0.5 average polymorphism and a value higher than 0.5 a very polymorphic locus (BOTSTEIN *et al.*,, 1980).

Cluster analysis was performed to generate a dendrogram using the unweighted pair group method with arithmetic averages (UPGMA) with PopGen 32 software. Principal coordinate analysis (PCoA) was further analyzed using PAST software (HAMMER *et al.*,, 2001). The analysis of molecular variance (AMOVA) was performed by use of the software package GenAlex 6.501 (YEH and YANG, 1999) to partition the total molecular variance among and within accessions.

RESULTS

A total of 196 individuals of saffron accessions from 20 geographically separate populations from Iran, Spain and Turkey were analyzed with 30 iPBS primers. Of those, 28 primers (93%) displayed reproducible and scorable bands across all the samples (Table 2). These primers amplified a total of 189 alleles. Among them, 179 alleles (94.7%) produced polymorphic loci and 10 alleles (5.3%) were monomorphic. The size of reproducible bands ranged from 0.1 to 3.0 kp. Primers 2226 and 2229 had the most bands with 10 bands and primers 2389 and 2395 had the smallest band with 3 bands (Table 2). To evaluate genetic structure among studied saffron accessions, genetic distance (GD) values were estimated (Table 3). The results showed that GD values ranged from 0.074 to 0.411 with an average of 0.286. The results of phylogenetic trees based on UPGMA method showed that 20 saffron accessions were assigned into four major clusters at the genetic similarity of 0.40 (Fig. 1). The accessions Gorgan and Mashhad were placed in the cluster (II). On the other hand, the cluster results could narrowly distinguish Turkish and Spanish accessions from others and they were classified separately in distinct clusters III and IV respectively. Associations among 20 studied saffron accessions were also resolved by principal coordinate analysis (PCoA) based on a genetic similarity matrix (Fig. 2). In the diagram generated by PCoA, four main groups were shown. Analysis of molecular variance (AMOVA) was conducted to partition the genetic variation among and within groups. The results of AMOVA demonstrated that the greatest variation can be attributed to within populations (74%).

Overall, the results of PCoA both showed a similar cluster result and emphasized that cluster analysis is more appropriate for revealing genetic relationship of saffron accessions. The first three principal axes explained 61.33% of the total molecular variation, which accounted for 39.11%, 15.19% and 7.03% of the total variation, respectively (Fig. 2).

Table 3. Genetic distance (GD) values among the studied C. sativus accessions calculated by iPBS marker

	1																		
2	0.107	2																	
3	0.169	0.099	3																
4	0.155	**0.074**	0.090	4															
5	0.145	0.130	0.177	0.146	5														
6	0.171	0.114	0.133	0.116	0.094	6													
7	0.152	0.102	0.159	0.109	0.077	0.099	7												
8	0.165	0.096	0.166	0.114	0.120	0.107	0.081	8											
9	0.158	0.117	0.191	0.138	0.115	0.123	0.116	0.131	9										
10	0.130	0.147	0.229	0.188	0.137	0.161	0.152	0.171	0.121	10									
11	0.301	0.220	0.209	0.205	0.291	0.231	0.257	0.230	0.214	0.303	11								
12	0.154	0.149	0.195	0.172	0.182	0.186	0.162	0.163	0.114	0.154	0.184	12							
13	0.187	0.117	0.143	0.116	0.186	0.154	0.153	0.160	0.113	0.172	0.133	0.125	13						
14	0.167	0.130	0.157	0.125	0.199	0.180	0.177	0.175	0.173	0.187	0.151	0.155	0.093	14					
15	0.152	0.181	0.250	0.214	0.134	0.193	0.144	0.193	0.119	0.125	0.294	0.140	0.193	0.176	15				
16	0.149	0.173	0.252	0.194	0.169	0.198	0.171	0.166	0.172	0.106	0.271	0.171	0.149	0.153	0.127	16			
17	0.195	0.200	0.257	0.259	0.216	0.204	0.234	0.226	0.200	0.129	0.266	0.203	0.202	0.216	0.213	0.163	17		
18	0.192	0.196	0.198	0.213	0.185	0.176	0.201	0.211	0.173	0.135	0.279	0.197	0.195	0.187	0.199	0.201	0.146	18	
19	0.179	0.193	0.261	0.228	0.142	0.176	0.183	0.211	0.188	0.153	0.288	0.189	0.195	0.197	0.180	0.181	0.155	0.150	19
20	0.390	0.306	0.280	0.291	0.354	0.305	0.310	0.323	0.359	0.405	0.343	0.333	0.304	0.272	**0.411**	0.379	0.342	0.301	0.343

1: Roshtkhar, 2: Yazd, 3: Kerman, 4: Birjand, 5: Bardaskan, 6: Esfehan, 7: Fars, 8: Gonabad, 9: Ferdos; 10: Bejestan, 11: Torbat heydarieh, 12: Zaveh, 13: Ghaen, 14: Kashmar, 15: Torbat jam, 16: Neyshaboor, 17: Gorgan, 18: Mashhad, 19: Izmir, 20: Barcelonan

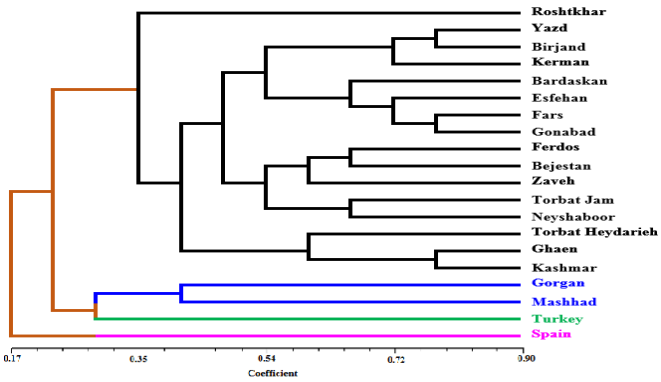

Fig 1. UPGMA dendrogram of 20 saffron accessions collected from different regions of Iran, Spain and Turkey based on iPBS fingerprinting data

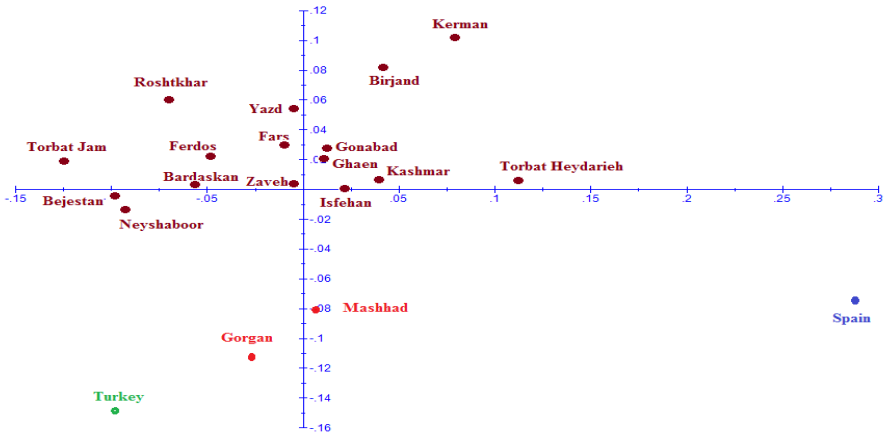

Fig 2. Relationships among saffron accessions visualized by principal coordinate analysis (PCoA)

DISCUSSIONS

The average of polymorphic bands per primer was 6.4 bands. Many earlier studies on *C. sativus* reported that there were genetic diversities among saffron accessions. For example, the average of polymorphic bands in the studies of BABAEI *et al.*, (2014) using SRAP, CAIOLA *et al.*, (2004) using RAPD, BEIKI *et al.*, (2010) using RAPD, MORAGA *et al.*, (2010) using ISSR, SIK *et al.*, (2008) using RAPD and ISSR, NEMATI *et al.*, (2012) using SSR, EROL *et al.*, (2014) using AFLP and NAMAYANDEH *et al.*, (2013) using SSR was 7.74, 2.1, 3.8, 4.8, 11, 2.1, 30.8 and 1.7, respectively. Our results indicated that not only there were genetic variations in the studied saffron accessions, but also the iPBS markers were an efficient technique for evaluating the genetic diversity among *C. sativus* accessions. The excellent efficiency of iPBS markers for

identifying high genetic diversity among plants have been reported by several researchers such as MEHMOOD *et al.,* (2013); FANG-YONG and JI-HONG (2014) and GUO *et al.,* (2014).

In order to further evaluate the performance of the iPBS markers and assess the genetic diversity among the varieties, the parameters of Shannon's information index (I) and genetic diversity (h) were calculated (Table 2). The average of parameters (I) and (h) was 0.483 and 0.841 respectively. These results indicated that 28 iPBS markers used in this study revealed a wide range of genomic DNA diversity among the studied saffron accessions. On the other hand, the polymorphic information index (PIC) values, as the marker index are used to estimate the discriminating ability and informativeness of utilized markers and measure the efficiency of polymorphic loci in revealing genetic diversity among the varieties (EROL *et al.,*, 2014; GUO *et al.,*, 2014). In this study, PIC values ranged from 0.407 to 0.953 with an overall average of 0.824 per primer (Table 2). The average PIC values in this study is higher than those reported in saffron accessions by BABAEI *et al.,* (2014) with SRAP (0.15), EROL *et al.,* (2014) with AFLP (0.34), NEMATI *et al.,* (2012) with SSR (0.34) and NAMAYANDEH *et al.,* (2013) with SSR (0.33). Primers 2298, 2229 and 2393 with 0.953, 0.943 and 0.943 PIC, respectively were identified as the most informative primers. The results revealed not only these loci were highly informative but also iPBS markers are particularly valuable in determining genetic diversity and relationships. Similarly, EROL *et al*. (2011, 2014) studied genetic diversity among *C. sativus* and introduced primers with the highest PIC values as the greatest discriminatory primers.

According to GD values, the most closely related two accessions were Yazd-Iran and Birjand-Iran, and the most genetically distant two accessions were Barcelona-Spain and Torbat Jam-Iran. Also, it clear that Spanish accession (average of 0.334) in comparison with Turkish accession (average of 0.192) had more genetic distance with Iranian accessions. This can be related to geographical distance among countries. Spain is far from Iran compared to Turkey, so we expected that the genetic distance between Spanish and Iranian accessions be larger than Turkish and Iranian accessions. Similarly, NAMAYANDEH *et al.,* (2013) estimated genetic distance values between 0.09 and 0.66 among Iranian *C. sativus* genotypes. Also SIK *et al.,* (2008) stated that genetic distance values among *Crocus* L. species from western Turkey ranged between 0.06 and 0.52. CAIOLA *et al.,* (2004) estimated GD values between 0.47 and 0.77 among *C. sativus* accessions that were collected from different countries.

Moreover, the result of private band analysis showed that only accession Gorgan had private band (10 of 179 polymorphic bands, about 0.06%) (Table 1), but, approximately 94 percent of polymorphic bands had common bands among saffron accessions. Genetic distance results confirmed the similarity of genetic structure among saffron accessions. Also, EROL *et al.,* (2014) reported that the genetic similarity coefficients among the 26 Crocus specimens varied widely, between 0.29 and 0.86. The close relationships among saffron accessions revealed in this study can be due to vegetative propagation, artificial selection by humans. These results are in agreement with those obtained by NAMAYANDEH *et al.,* (2013) and BABAEI *et al.,* (2014).

This was further confirmed by the high genetic distance of Spanish and Turkish accessions with Iranian accessions. In the study, similar to BABAEI *et al.,* (2014) results, the most of saffron accessions were included in their major groups. They concluded that their studied materials had similarly genetic structure. Overall, the results of cluster analysis indicated not only these groupings were in congruence with their geographical affinities but also it emphasized that iPBS technique was a very reliable method to measure genetic relationships and investigate the genetic structure among saffron accessions originating from different geographical sites. ANDEDEN *et al.,*

(2013) and BARÁNEK *et al.,* (2012) believed that retrotransposon markers have the good abilities for evaluating genetic relationships among plants and the obtained results are very reliability.

In this study, we found some level of genetic variation among populations (26%) that could be related to different saffron accessions from different geographical locations of Iran, Spain and Turkey. Results from this research proved that iPBS marker system can properly detect genetic differences not only at inter-specific level but also at intra-specific level. Our results are in agreement with those obtained by NAMAYANDEH *et al.,* (2013).

CONCLUSION

This study provided significant insights on the genetic diversity present in *C. sativus*. It confirmed that the iPBS marker is a simple, informative and suitable approach for the evaluation of genetic diversity and phylogenetic analysis among saffron accessions. The results of this study demonstrated that there was genetic diversity among *C. sativus* collected from various geographical regions of Iran, Spain and Turkey. Our data should be a valuable resource for breeding and genetic conservation programs in saffron growing regions.

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DIVERZITET I FILOGENOJA UZORAKA ŠAFRANA (*Crocus sativus* L.) NA OSNOVU IPBS MARKERA

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Izvod

Ovo istraživanje je imalo za cilj utvrđivanje genetičke raznovrsnosti i filogenetsku strukturu 196 uzoraka šafrana iz 20 geografski udaljenih područja Irana, Spanije i Turske, korišćenjem iPBS markera. 28 prajmera je amplifikovalo 179 polimorfnih alela sa prosečnim brojem od 6,4 trake po prajmeru. Prosečna vrednost *Shannon*-ovog indeksa, genetičke distance i diverzitet gena iznosili su 0.483, 0.286 i 0.841. Prajmeri 2298, 2229 i 2393 su bili najinformativniji u ovom radu. Filogenetsko stablo grupisalo je 20 uzoraka šafrana u četiri klastera u skladu sa njihovim geografskim poreklom, što je potvrđeno i PCA analizom. Možemo zaključiti da su iPBS markeri, kao jeftini i visoko efikasni, pogodni za proučavanje genetičkog diverziteta i filogenetske analize uzoraka šafrana iz različitih geografskih područja.

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