



Original article

Deciphering genetic diversity analysis of saffron (*Crocus sativus* L.) using RAPD and ISSR markers



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ABSTRACT

The existence of genetic diversity in *Crocus sativus* has globally remained a mystery till date. The study investigated PCR based DNA amplification profile of saffron using ISSR and RAPD based primers. A total of 38 amplicons were generated by ISSR primers in the range from 7 to 12 with an average of 9.50 bands per primer. 20 bands were found to be polymorphic and 18 were monomorphic with an average percentage of polymorphism as 52.48%. RAPD based amplification revealed a total 161 amplicons, 107 as polymorphic and 54 as monomorphic with an average percentage of polymorphism as 66.44%. Cumulative results of RAPD and ISSR demonstrated that Nei-Li's similarity index ranged between 0.70 and 0.97. The results of AMOVA has revealed 9% of variance among populations and 91% of variance within populations, Φ_{PT} was found as 0.089, which indicates existence of genetic differences though limited. In conclusion, the results indicate that saffron accessions are minimally genetically differentiated, which could be capitalized in future breeding programmes to ameliorate this precious crop.

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1. Introduction

Crocus sativus (Family- *Iridaceae*) is a very fabulous crop and is the source of world's costliest spice in the form of saffron which is very fragile in nature that needs intense care during ploughing and harvesting (Shokrpour 2019). It is an exotic crop of chief economic importance possessing various cultural and therapeutic properties recognized since ancient civilizations (Namayandeh et al. 2013). Among the world's total saffron production of 205 tons, Iran contributes 160 tons (~80%), Jammu and Kashmir contributes around 8–10 tons (~5%), Greek 4–6 tons (~3%), Morocco

0.8–1 ton (~0.5%), Spain 0.3 to 0.5 ton (~0.25%) and rest is contributed by other countries (3,22,43).

The mysterious origin, complex taxonomy and identification of elite species has led researchers to study phenotypic, cytological, biochemical (Transcriptome, proteome and metabolome) and molecular aspects of *Crocus* genus. It is believed that biological origin of *C. sativus* is *C. catwrichtianus* or *C. thomasi* (Ahmad et al. 2011; Beiki et al. 2010; Caiola and Canini 2010; Poma et al. 2012). Many researchers studied phenotypic differences included accessions with larger styles, stamens, increased number of stigmas, tepal colour intensity, bigger corm size, more flowers and viability. Although, few reports mentioned phenotypic variants of saffron possessing increased number of stigmas. Nevertheless, because of its sterile nature these variations are not always heritable. The lack of genomic variation via corm propagation, genetic erosion due to urbanization of cultivable saffron land mounts up the limited genetic variations (Ali et al. 2013; Caiola and Canini 2010; Fernández, 2006, Fernández et al., 2011; Keify and Beiki 2012). There exists an enigma in the literature data whether *C. sativus* is monomorphic or polymorphic, however majority of researchers have proved existence of limited genetic variability

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in this high-valued crop with possibility of genetic improvement (Beiki et al. 2010; Busconi et al. 2018; Namayandeh et al. 2013; Zheng et al. 2013). The cross talk between morphological characters versus genetic diversity is not the sole link because environmental and geographical factors also control their expression (Boulli et al. 2001; Degani et al. 1998; Fernández et al., 2011; Rubio-Moraga et al. 2009).

Previously different molecular markers have been used to assess genetic diversity of saffron in different regions of the world including Random amplified polymorphic DNA-RAPD (Ali et al. 2013; Beiki et al. 2011; Caiola and Canini 2010; Imran et al. 2010; Keify and Beiki 2012; Qadri et al. 2012; Zheng et al. 2013), Inter Simple Sequence Repeat-ISSR (Rubio-Moraga et al. 2009; Zheng et al. 2013), SSR-Simple Sequence Repeat (Namayandeh et al. 2013; Nemati et al. 2012; Zheng et al. 2013), Amplified Fragment Length Polymorphism-AFLP (Caiola and Canini 2010; Fernández et al., 2011; Nazzal et al. 2011; Siracusa et al., 2013; Zubor et al. 2004), Retero-transposons (Alavi-Kia et al. 2008), single nucleotide polymorphism-SNP (D'Agostino et al. 2007; Fernández et al., 2011), and expression sequence Tags-EST (D'Agostino et al. 2007). Some of the earlier researchers have conducted studies to explore genetic diversity of saffron from various areas limited to only Kashmir and results have indicated existence of significant level of polymorphism (Imran et al. 2010; Qadri et al. 2012), also an earlier study based on karyogram studies has reported that *Crocus sativus* L. 'Kashmirianus' a variety which is not genetically similar to usual *Crocus sativus* L. (Caiola and Canini 2010).

Saffron cultivation of Jammu and Kashmir, India dates back to antiquity and constitutes 16% of agricultural income. The Jammu and Kashmir region, India grows saffron over an area of 3785 ha. Within J&K, there are four districts which are climatically more favorable for saffron cultivation i.e. Pulwama, Budgam, Kishtwar and Srinagar. The production area of each district varies from one another, Pulwama contributes 3200 ha of land, Budgam 300 ha, Srinagar 165 ha and Kishtwar ~120 ha. Though, previous studies have focused on genetic diversity of *C. sativus* pertaining to only Kashmir region (Ali et al., 2013; Qadri et al., 2012). However, no studies were conducted towards genetic diversity of saffron across whole Jammu and Kashmir. Therefore, the current study attempted to evaluate the existence of genetic diversity in major saffron growing regions of Jammu and Kashmir (India) using PCR based molecular markers i.e. ISSR and RAPD.

2. Materials and methods

2.1. Plant material collection and authentication

Leaf samples of *Crocus sativus* L. were collected from four major saffron producing districts of Jammu and Kashmir, India in biological replicates (n = 2) From Pulwama district, the samples were collected from four places namely Pampore Khrew (PAMKHR), Pampore Konibal (PAMKON), Pampore Khankagbagh (PAMKBG), Pampore Samboora (PAMSAM). Similarly, from Srinagar district the samples were collected from two saffron growing regions i.e. Balhama (SGRBAL) and Mehjoor Nagar (SGRMN). Also, from Kishtwar region of Jammu, samples were collected from Berwar (KISBER), Hatta (KISHAT) and Kunda (KISKUN). While as in Budgam district, saffron grows majorly in Nagam area and it was collected accordingly. The samples were immediately immersed in liquid nitrogen and maintained at sub-zero temperature until DNA extraction. The authenticated specimens were deposited at Centre for Biodiversity and Taxonomy, University of Kashmir herbarium (KASH) under assigned voucher specimen numbers.

2.2. Molecular characterization

Genomic DNA extraction and purification: Extraction and purification of total genomic DNA was carried using slightly modified earlier methods (Chikkaswamy and Prasad 2012). The dried DNA pellet were re-dissolved in 0.5 ml TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA), followed by addition of 5 µl RNase A (2 mg/ml) and incubated for 60 min at 37 °C. DNA was further purified using phenol: Chloroform: Isoamyl alcohol (25:24:1)

2.3. Quantification and purity check of extracted DNA

DNA quantification, purity and quality check was determined by spectrophotometer (Chemito Technologies) using standard protocols (Ahmed et al. 2013; Devi 2005; Ibrahim 2011; Nwangburuka et al. 2011; Shankar et al. 1970) and the purified DNA was stored at –20°C till further analysis.

2.4. Polymerase chain reaction amplification

The PCR amplification of target DNA was carried out initially using 8 synthesized ISSR primers and only 4 primers were selected because they gave clear, reproducible and scorable bands. Similarly, 20 arbitrary (10-mer) RAPD primers of group A, B, C, D, E, F, G, M, N, T (Operon Technology, USA) were initially screened for detection of DNA polymorphism in 10 different samples of *Crocus sativus* L. Kashmirianus. However, only 15 primers showed high resolution, reproducibility, distinctness after repeating the same procedure twice. The amplified PCR products were separated on 1.5%(w/v) agarose gel.

2.5. Molecular data collection and analysis

The repeatable and unambiguous RAPD and ISSR amplification products (bands) which showed up during two replicated PCR amplifications were considered for scoring manually and were assigned '1' or '0' for presence or absence of bands respectively. The banding pattern derived from all 19 primers were used to marker DNA banding characteristics such as total number of bands (TB), number of polymorphic bands (PB), number of monomorphic bands (MB), percentage of polymorphic bands (PPB), number of monomorphic bands (MB). The discriminatory power of each marker primers was further calculated by four parameters [202–203] including polymorphic information content (PIC), effective multiplex ratio (EMR), marker index (MI), resolving power (RP) as per already existing literature (Varshney et al. 2007; Milbourne et al. 1997).

The genetic data matrix generated from scoring bands was utilized for analysis using MVSP 3.1 software (Kovach 2007) to generate pairwise genetic similarity matrices by Nei-Li's coefficients (Nei and Li 1979). The genetic closeness of all samples were analyzed via UPGMA and phylogenetic tree (dendrogram) was created accordingly. Furthermore, GenAlex 6.5 excel add-Ins software (Peakall and Smouse 2006; Peakall and Smouse 2012) was used for measurement of genetic diversity indices i.e. Nei's genetic distance (NeiGD), Shannon's Index (I), principal component analysis (PCA) was determined based on combined data of RAPD and ISSR similarity matrices and Mantel test was performed (99 permutations) to estimate the correlation between RAPD and ISSR using genetic distances based on matrix data of both markers and correlation coefficient (r) was determined.

2.6. Statistical analysis

All the measurements were done in triplicates and results are expressed as mean ± SD. The analysis of variance was performed

(ANOVA) by using Origin9 software (Origin Lab Corporation, Northampton MA, USA) and Graph Pad Prism 5.01 (Graph Pad Software, San Diego, CA, USA). Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) was also used for data analysis and interpretation. P values < 0.05 were considered statistically significant and P < 0.01 considered as very significant.

3. Results

3.1. Quality check and quantitative estimation of isolated DNA

The yield of DNA in each *Crocus sativus* sample was calculated (Table S1). The highest DNA yield was recorded in SGRMJN sample with $2715 \pm 1.8 \mu\text{g/ml}$ which was followed by BUDNAG, SGRBAL samples with DNA concentrations as $2715 \pm 1.8 \mu\text{g/ml}$ and $2500 \pm 2.35 \mu\text{g/ml}$ respectively (Table S2). The least yield was found in KISKUN sample as $247 \pm 1.0 \mu\text{g/ml}$. Furthermore, absorbance ratio 260/280 was found in the range of 1.77–1.83 which falls in the ratio recommended for pure DNA preparations i.e. 1.8–2.0. The presence of discrete and unbreakable DNA bands found during electrophoresis reflects purity of isolated genomic DNA which allowed further PCR amplification.

3.2. Molecular marker-based polymorphism

3.2.1. ISSR profiling

Samples of *Crocus sativus* L. Kashmirianus were analyzed using 8 ISSR primers and out of which only 4 produced clear, scorable and reproducible bands and rest of the four ISSR primers didn't show any proper amplified bands i.e. (ISSR169, ISSR170, ISSR171&ISSR172). There were total 38 amplicons/loci generated by these ISSR primers in the range from 7 to 12 with an average of 9.50 bands per primer (Table 1). The highest number of total bands produced by ISSR-8 were 12, followed by ISSR-1(9) and ISSR-2 (10), whereas ISSR-9 amplified least number (7). Furthermore, out of total 38 amplicons, 20 were found to be polymorphic and 18 were monomorphic with an average percentage of polymorphism as 52.48%. The ISSR profile from various representative gels are represented (Fig. 1), the percentage of polymorphism in ISSR-1, ISSR-2,

ISSR-8, ISSR-9 primers was found as 44.44%, 50.0%, 58.33%, 57.14% respectively. The size of these amplified bands varied between mean size of 150–2000 bp.

The discriminatory potential of markers were assessed using PIC, MI, RP parameters and therefore discriminatory powers of each ISSR primer were calculated. The highest resolving power (RP) of 3.40 was found with ISSR-8 primer with an average value of RP per primer as 2.0. The mean Polymorphism information content (PIC) value of each primer was calculated using average PIC value of each loci. The ISSR primers have showed PIC value ranged between 0.11 and 0.25 with an average value of 0.16 per primer. PIC of 0.25 was found to be highest in ISSR-8 and is statistically significant ($p < 0.05$) as compared to other primers. Further, marker index (MI) of each primer was calculated, the highest MI of 1.07 was showed by ISSR-8 primer with an average value of 0.59 per primer. The effective multiplex ratio (EMR) was found in the range between 3.02 and 4.33 with an average value of 3.51 per primer, highest EMR of 4.33 was found with ISSR-8 primer. We have found that among all ISSR primers used in the current study, the highest percentage of polymorphism, total number of bands, PIC, MI, EMR and RP values were observed with ISSR-8 primer and the values are statistically significant ($p < 0.05$) compared to other primers. Nei's genetic distance (NeiGD) and Shannon's Index (I) were found as 0.088 and 0.248 respectively.

The Nei-Li's similarity coefficient was calculated using MVSP 3.1 software which demonstrated that similarity index ranged between 0.621 and 1 with mean value as 0.908 (Table 2). The genetic closeness of all 10 *C. sativus* samples were analyzed via UPGMA algorithm i.e. Unweighted Pair-Group Method of Arithmetic average cluster analysis and dendrogram was created based on genetic distance obtained from ISSR markers (Fig. 2A). The molecular dendrogram has revealed two distinct clusters which joined to form a major cluster at 0.62 level of similarity. The cluster 2 is composed of six sample accessions (PAMKHR, PAMKON, SGRBAL, PAMSAM, BUDNAG, KISHAT) joining to form a single cluster at 0.95 level of similarity and cluster 1 is composed of two sub clusters i.e. 1a and 1b joining at 0.62 level of similarity.

Further, sub cluster 1b is composed of three accessions (KISBER, KISKUN & SGRMJN) which joined at similarity level of 0.79. Cluster

Table 1
Details of RAPD/ISSR primers and marker parameters used for the study of *Crocus sativus* L. Kashmirianus molecular characterization.

S No.	Primer Name	Primer Sequence	TB	MB	PB	PPB (%)	RP	MI	PIC	EMR
1.	ISSR-1	CACACACACACAGG	9.00	5.00	4.00	44.44	1.20	0.38	0.11	3.60
2.	ISSR-2	CTCTCTCTCTA.C	10.00	5.00	5.00	50.00	2.00	0.46	0.15	3.10
3.	ISSR-8	GAGAGAGAGAGAGG	12.00	5.00	7.00	58.33	3.40	1.07	0.25	4.33
4.	ISSR-9	GTGTGTGTGTGTGG	7.00	3.00	4.00	57.14	1.40	0.44	0.15	3.02
Total			38.00	18.00	20.00					
Average Primer			9.50	4.50	5.00	52.48	2.00	0.59	0.16	3.51
1.	OPG09	CTGACGTCAC	10.00	3.00	7.00	70.00	4.41	1.19	0.27	4.41
2.	OPT06	CCGICCTGA	10.00	4.00	6.00	60.00	4.20	0.82	0.39	2.13
3.	OPF03	CCTGATCACC	11.00	4.00	7.00	63.64	2.20	0.60	0.19	3.23
4.	OPM13	TCAGTCCGGG	10.00	5.00	5.00	50.00	1.60	0.51	0.13	4.00
5.	OPA09	GGGTAACGCC	12.00	5.00	7.00	58.33	2.00	0.76	0.20	3.90
6.	OPB05	TGGCCCTTC	11.00	6.00	5.00	45.45	1.20	0.41	0.09	4.36
7.	OPD18	GAGAGCCAAC	9.00	2.00	7.00	77.78	3.60	1.43	0.28	5.20
8.	OPE20	AACGGTGACC	11.00	2.00	9.00	81.82	3.20	1.30	0.25	5.15
9.	OPN16	AAGCGACCTG	12.00	4.00	8.00	66.61	3.00	0.93	0.19	4.90
10.	OPA17	GACCGCTTGT	13.00	4.00	9.00	69.23	2.80	0.62	0.17	3.69
11.	OPT01	GGGCCACTCA	10.00	2.00	8.00	80.00	2.40	1.10	0.20	5.60
12.	OPC02	GTGAGGCGTC	10.00	2.00	8.00	80.00	4.20	1.40	0.28	4.99
13.	OPC08	TGGACCGGTG	11.00	1.00	10.00	90.91	5.00	2.13	0.35	6.09
14.	OPB10	CTGCTGGGAC	12.00	5.00	7.00	58.33	5.40	0.80	0.20	4.03
15.	OPD20	ACCCGGTCAC	9.00	5.00	4.00	44.44	4.40	1.33	0.26	5.15
Total			161.00	10.700						
Average Primer			10.73	7.13		66.44	3.31	1.02	0.23	4.46

Note: TB- total number of bands, MB- monomorphic bands, PB- polymorphic bands, PPB- percentage of polymorphism, RP-resolving power, MI-marker index, PIC-polymorphic information content, EMR-effective multiplex ratio.

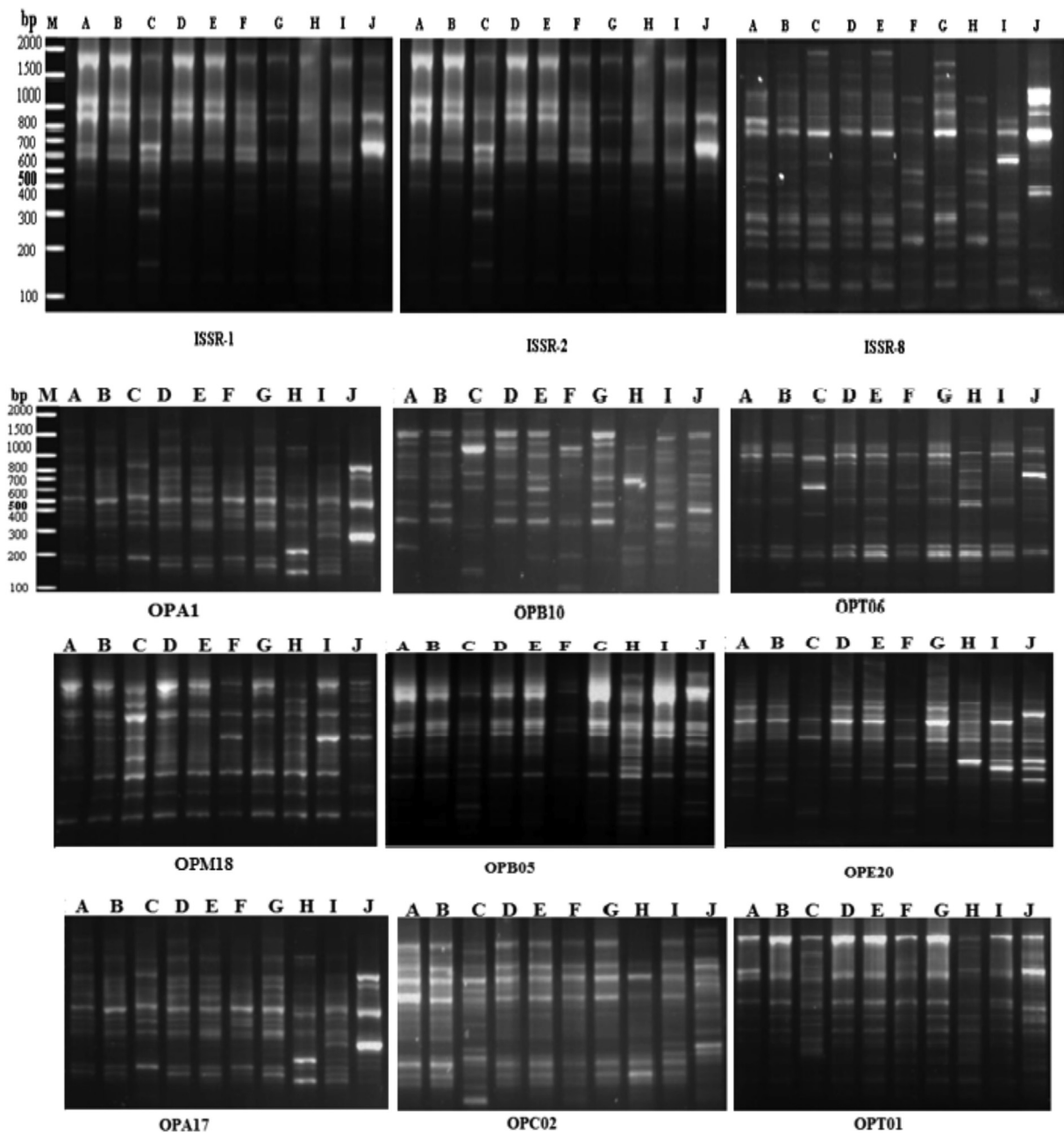


Fig. 1. Selected ISSR and RAPD gel profiles of *Crocus sativus* L. Kashmirianus accessions.

Table 2
Nei-Li's similarity matrix among accessions of *Crocus sativus* based ISSR data.

	PAMKHR	PAMKON	PAMKBG	SGRBAL	PAMSAM	KISBER	KISHAT	KISKUN	SGRMJN	BUDNAG
PAMKHR	1.00									
PAMKON	1.00	1.00								
PAMKBG	0.583	0.583	1.00							
SGRBAL	1.00	1.00	0.583	1.00						
PAMSAM	1.00	1.00	0.583	1.00	1.00					
KISBER	0.816	0.816	0.683	0.816	0.816	1.00				
KISHAT	0.963	0.963	0.609	0.963	0.963	0.809	1.00			
KISKUN	0.836	0.836	0.681	0.836	0.836	0.708	0.83	1.00		
SGRMJN	0.857	0.857	0.708	0.857	0.857	0.735	0.852	0.982	1.00	
BUDNAG	0.982	0.982	0.571	0.982	0.982	0.8	0.945	0.821	0.842	1.00

Note: I = Shannon's Information Index; Nei GD= Nei's Genetic Distance.

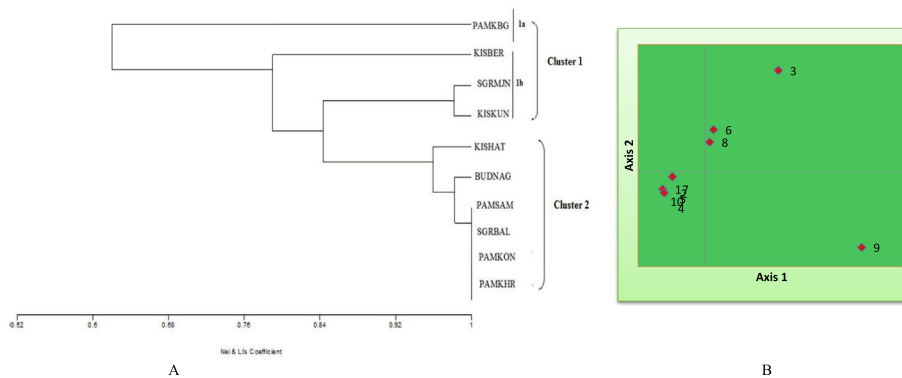


Fig. 2. A) Dendrogram illustrating genetic relationship among different samples of *Crocus sativus* L. Kashmirianus using ISSR-UPGMA clustering method. B) Two-dimensional principal coordinate analysis of *Crocus sativus* accessions based on ISSR markers.

1a was found to be most distinct from other sample accessions and was found joining with other clusters at 0.62 level of similarity. The clustering report and dendrogram illustrates that the most closely related genotypes with 100% similarity index were PAMKHR, PAMKON, SGRBAL & PAMSAM followed by BUDNAG, KISHAT, SGRMKN & KISKUN with similarity index of 0.97. While as KISBER accession has showed similarity index of 0.79 with cluster 2, SGRMKN & KISKUN. In order to obtain relationship between all ten genotypes based on ISSR genetic distance data, the principal coordinate analysis (PCoA) was derived which showed that the first three axes or principal coordinates accounted for total of 89.99% of genetic similarity variance i.e. Axis 1 = 48.52%, Axis2 = 27.66% and Axis 3 = 12.81%. We found distribution of genotypes using two dimensional PCoA in the same way as obtained by dendrogram (Fig. 2B).

3.2.2. RAPD profiling

The screening of clefFar bands during the initial phase illustrated that out of 19 RAPD primers tested, only 15 produced clear and repeatable bands while as 4 primers didn't not produce anticipated amplification viz. OPC04, OPG09, OPT06, OPG17. These 15 primers generated a total of 161 bands across all the accessions in the range from 9 to 13 with an average of 10.73 bands per primer (Table 1). The highest number of total bands were produced by OPA17 as 13, followed by OPA9, OPN16, OPB10 with 12 bands, also 10 bands were found in each of the primer viz. OPG09, OPT06, OPM18, OPT01, OPC02. The least number of bands were found in OPD18 & OPD20 as 9. Furthermore, out of total 161 amplicons (Fig. 1), 107 were found to be polymorphic and 54 were monomorphic with an average of 7.13 and 3.60 as polymorphic and monomorphic bands per primer. The average percentage of polymorphism was found as 66.44% with highest percentage of polymorphism shared by OPC08 (90.91%). Furthermore, greater than 80% of polymorphic bands were also showed by OPE20, OPT01 & OPC02. The RAPD based gel profile from various representative gels (Fig. 1), the percentage of polymorphism among RAPD primers varied between 44.44% and 90.91%. The size of the amplified bands varied with mean size of 150–2000 bp. The discriminatory potential of markers are usually being assessed using PIC, MI, RP parameters and therefore discriminatory powers of each ISSR primer was calculated (Table 1).

The highest resolving power (RP) of 5.40 was found in OPB10 primer with an average value of RP per primer as 3.31. The mean Polymorphism information content (PIC) value of each primer was calculated using average PIC value of each loci. The RAPD primers have showed PIC value ranged between 0.13 and 0.39 with an average value of 0.23 per primer. PIC value of 0.39 was found to be highest in OPT06 which is statically significant ($p < 0.05$) value as

compared to other primers. The marker index (MI) of each primer was calculated and highest was value of 2.13 was found in OPC08 with an average of 1.02 per primer. The effective multiplex ratio (EMR) was found in the range between 2.13 and 6.09 with an average value of 4.46 per marker. We have found in the current study that among all RAPD primers used, the higher percentage of polymorphism, MI, EMR and RP values were observed with OPC-08 primer ($p < 0.05$). Also, OPC-08, OPT06, OPC02, OPD20 have shown significant levels of percentage of polymorphism, PIC, MI, EMR and RP as compared to other markers used. All 10 *C. sativus* samples under investigation were divided into three populations based on geographical locations i.e Population-1(PAMKHR, PAMKON, PAMSAM, PAMKBG, SGRBAL), Population-2 (KISBER, KISHAT, KISKUN) and Population-3 (SGRMKN and BUDNAG). The Nei's genetic distance (NeiGD) and Shannon's Index (I) were found as 0.172 and 0.194 respectively.

The Nei-Li's similarity coefficient was calculated using MVSP 3.1 software which demonstrated that similarity index ranged between 0.71 and 1 with mean value as 0.87 (Table 3). The genetic closeness of all 10 *C. sativus* samples were analyzed via UPGMA algorithm and dendrogram was created based on genetic distance obtained from 15 RAPD markers (Fig. 3A). The molecular dendrogram has revealed two distinct clusters which joined to form a major cluster at 0.71 level of similarity.

The cluster 2 is composed of three sub clusters i.e. 2a, 2b and 2c joining to form a single cluster at 0.88 level of similarity and cluster 1 is composed of two sub clusters i.e. 1a and 1b joining at 0.71 level of similarity. Also, sub cluster 1b is composed of three accessions (KISBER, KISKUN & KISHAT) which joined at similarity level of 0.87. Cluster 1a was found to be most distinct from other sample accessions and was found joining with other clusters at 0.71 level of similarity. The clustering report and dendrogram illustrated that the most closely related genotypes with 0.95% similarity index were PAMSAM, BUDNAG, SGRBAL, PAMKHR & PAMKON followed by other accessions with average similarity index of 0.82. In order to ascertain relationship between accessions based on RAPD genetic distance data, the principal coordinate analysis (PCoA) was derived which showed that the first three axes or principal coordinates accounted for total of 89.99% of genetic similarity variance i.e. Axis 1 = 48.52, Axis2 = 27.66 and Axis 3 = 12.81. We have found good correlation between genotypes using two dimensional PCoA in the same way as obtained by using dendrogram (Fig. 3B).

4. Discussion

Crocus sativus L. is a crop of chief economic importance (Ríos et al. 1996) as it being a source of world's costliest spice known as saffron. The immense properties of this spice marks it as a red

Table 3
 Nei-Li's similarity matrix among accessions of *Crocus sativus* L. Kashmirianus based on RAPD data.

	PAMKHR	PAMKON	PAMKBG	SGRBAL	PAMSAM	KISBER	KISHAT	KISKUN	SGRMJN	BUDNAG
PAMKHR	1.00									
PAMKON	0.946	1.00								
PAMKBG	0.673	0.68	1.00							
SGRBAL	0.925	0.947	0.727	1.00						
PAMSAM	0.907	0.929	0.701	0.955	1.00					
KISBER	0.781	0.79	0.733	0.813	0.824	1.00				
KISHAT	0.845	0.856	0.707	0.875	0.876	0.871	1.00			
KISKUN	0.775	0.784	0.721	0.797	0.815	0.828	0.861	1.00		
SGRMJN	0.857	0.869	0.758	0.908	0.89	0.788	0.845	0.745	1.00	
BUDNAG	0.921	0.943	0.695	0.95	0.968	0.818	0.871	0.81	0.875	1.00

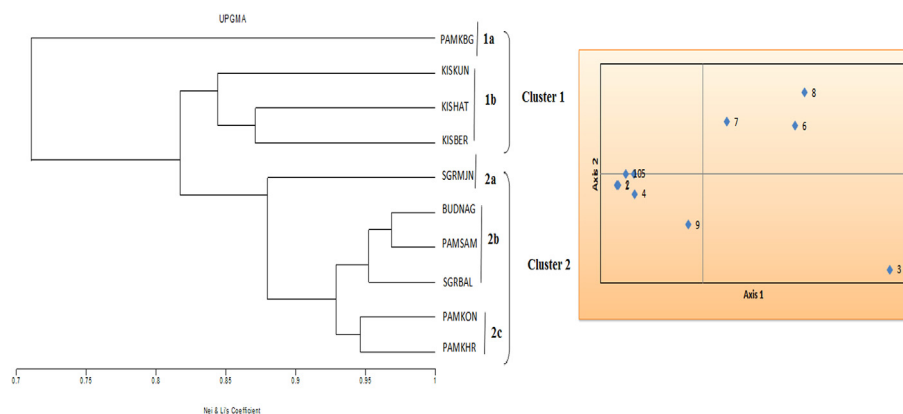


Fig. 3. A) Dendrogram illustrating genetic relationship among different samples of *Crocus sativus* L. Kashmirianus using RAPD-UPGMA clustering method. B) Two-dimensional principal coordinate analysis of *Crocus sativus* accessions based on RAPD markers.

Gold for any country capable of growing this evocative spice as its commercialization would fetch tangible profits, that would in turn help in uplifting the socio-economic conditions of people and would pave a way to leverage the economy. There are numerous properties attributed to this delicate golden crop ranged including therapeutic medicinal benefits and capacity to be an agent for pharmaceutical industry (Ferrence and Bendersky 2004; Hosseini et al. 2018; Mir et al. 2020; Nemati et al. 2014; Razak et al. 2017). However, there are multitude of challenges which the current saffron industry is facing and to overcome these challenges to an extent, biotechnological interventions could play a crucial role to ameliorate this precious crop at the molecular level such as disease, abiotic stress resistant cultivars or cultivars with superior chemical profiling etc.

The genetic relationship between different sample accessions of *Crocus sativus* L. Kashmirianus was therefore assessed using cumulative data of RAPD and ISSR analysis. As high quality, genomic DNA is a pre-requisite for majority of molecular biology studies including RAPD, AFLP, southern blotting, library construction of crop, forestry, medicinal plants etc. (Ahmed et al. 2013; Ibrahim 2011; Pathak et al. 2013) and DNA extraction from aromatic and herbal plants is usually cumbersome due to presence of large number of secondary metabolites. Therefore purity, quality and quantity of DNA obtained in this study indicated that this extraction method could be used for routine DNA extraction of *Crocus sativus* samples in a cost-effective manner.

The RAPD based profiles usually represent widely distributed regions of the genome and ISSR based profiles arise from microsatellite portions of the genome. Therefore PCR-RAPD-ISSR based methods involve those regions of the genome which are having different genome coverage as well as different evolutionary

histories (Kumar et al. 2014). The total number of RAPD and ISSR markers which were used in the current study in order to produce clear and reproducible bands were 19 which resulted in the generation of 199 bands of which 59.46% were polymorphic and rest as monomorphic. The existence of DNA polymorphism in *C. sativus* has been reported by earlier studies with varied percentage of polymorphism depending upon the primers used i.e. 0 %to 20.5% by Siracusa et al. (2013), 54% by Keify and Beiki (2012), 22% by Nemati et al. (2012). The average number of bands per marker was found as 10.47 and highest number of total bands were produced by OPA17 as 13, followed by OPA9, OPN16, OPB10, ISSR-8 with 12 bands, also 10 bands were found in each of the primer Viz.OPG09, OPT06, OPM18, OPT01, OPC02 & ISSR-2. The least number of bands were showed by ISSR-9 i.e. 7. The average number of bands were found as 10 in an early study reported by Keify and Beiki (2012). The mean values of discriminatory marker parameters were found as RP = 2.65, MI = 0.805, PIC = 0.195 and EMR = 3.98. Polymorphism information content (PIC) value estimates the informativeness of a marker and the value found by current study (0.195) is consistent with earlier studies by Nemati et al. (2014) and Keify and Beiki (2012) as 0.1–0.54 and 0.15–0.82 respectively. Based on the cumulative data of RAPD and ISSR the various genetic diversity indices were calculated i.e. Nei's genetic distance (NeiGD) and Shannon's Index (I) with values as 0.155 and 0.204 respectively (Table 4).

Although there are different methods of determining genetic similarity coefficients such as simple matching coefficient, Jaccard's similarity coefficient and Nei-Li's coefficient (Jaccard 1901; Kovach 2007; Lamboy, 1994). However, we preferred to determine Nei-Li's coefficient, this is because *C. sativus* is considered to be closely related with limited genetic relationship due to its triploid

Table 4
Genetic Diversity Indices analysed in *Crocus sativus* L. Kashmirianus populations using both RAPD and ISSR primers.

No. of Populations		I	Nei GD
3	Mean	0.204	0.155
	SE	0.012	0.070

Note: I = Shannon Index; Nei GD = Nei's Genetic Distance.

nature (Gedik et al. 2017; Zubor et al. 2004) and as per Lamboy (1994), for closely related organisms, Nei and Li's coefficient show less percent of bias in the interpretation than the simple matching and Jaccard's similarity coefficient. This is because Nei and Li's coefficient has direct biological meaning as it is an estimate of the expected proportion of amplicons shared by two samples as they had a common ancestor. Based on Nei-Li's similarity matrix (Table 4), the similarity coefficient varied between 0.97 and 0.70 and molecular dendrogram has revealed three distinct clusters which joined to form a major cluster at 0.70 similarity level (Fig. 4A). Cluster 1 consists of two accessions (KISKU and KISBER) which showed 0.80 similarity index. Cluster 2 was found comprised of two accessions with similarity index as 0.87. Also, cluster 3 was found composed of 2 sub clusters viz. 3a (BUDNAG, PAMSAM and SGRBAL) and 3b (PAMKON and PAMKHR) which joined together at 0.95 similarity index which is the closest level of relationship between these genotypes (Fig. 4B).

There is only one sample accession (PAMKBG) which did not form any cluster with other accessions and is matching with other genotypes at around 0.70 similarity level. The different levels of similarity index have been found in the earlier studies which indicated there exists genetic variation, although within the genotypes of *C. sativus* e.0.2593 to 0.6852 (Keify and Beiki 2012); 0.375 to 0.834 (Ali et al. 2013; Imran et al. 2010). Furthermore, in order to ascertain relationship between these ten genotypes based on genetic distance data of both RAPD and ISSR, the cumulative principal coordinate analysis (PCoA) was estimated which showed that the first three axes or principal coordinates accounted for total of 73.97% of genetic similarity variance i.e. Axis 1 = 35.94, Axis 2 = 23.44 and Axis 3 = 14.59. We have found distribution of genotypes using two dimensional PCoA in the same way as obtained by dendrogram and results indicated that genetic variability in *C. sativus* L. Kashmirianus was not always completely associated with geographical locations and thus our findings are matching with an earlier study which reported that geographical diversity, although is important but was not always the factor in determining the genetic divergence (Ali et al. 2013).

The results of AMOVA has revealed 9% of variance among populations and 91% of variance within populations, also a statistically significant value of Φ_{PT}/Φ_{IPT} ($p < 0.01$) was observed as 0.128

which indicates there exists genetic difference among the populations (Table 5). This is because if $\Phi_{IPT} > 0$, it means there exists genetic difference among the populations (Peakall and Smouse 2012). AMOVA is also calculated in previous studies (Anabat et al. 2020; Nemati et al. 2014) which have indicated that *Crocus sativus* showed higher levels of intra population variation as compared to variation among populations. In order to check correlation of markers, Mantel test (99 permutations) was performed to estimate the correlation between RAPD and ISSR using genetic distances based on matrix data of both markers and correlation coefficient (r) was determined. Pair-wise Mantel test was performed (Fig. 5) among genetic distance matrices obtained from RAPD and ISSR molecular markers and it showed statistically significant correlation between the two markers ($r = 0.634$ and $P = 0.02$). This indicates that there is a pattern of good association between the results achieved by ISSR and RAPD analysis in the discrimination of *C. sativus* genotypes.

Although various previous studies have been conducted to analyze the existence of molecular diversity in *C. sativus* species. However, there are conflicting conclusions drawn from these studies by the researchers; some illustrated that this crop is monomorphic at molecular level due to its triploid nature (Alsayied et al. 2014; Caiola et al. 2000; Rubio-Moraga et al. 2009; Zubor et al. 2004) while as other researchers mentions that a significant level of polymorphism does exist at DNA level (Agayev et al. 2007; Alavi-Kia et al. 2008; Ali et al. 2013; Beiki et al. 2010; Keify and Beiki 2012; Nemati et al. 2014; Nemati et al. 2012; Qadri et al. 2012; Torricelli et al. 2019). The results of current study indicates that there exists genetic variability and significant polymorphism among the accessions of *C. sativus* from different regions of Jammu and Kashmir using RAPD and ISSR primers and thus interpretation of our results are in sync with earlier researchers who studied DNA diversity of saffron localized to Kashmir (Ali et al. 2013; Imran et al. 2010).

The molecular markers being an effective way for obtaining information on existence of genetic diversity and to study population structure. Beiki et al. (2011) have suggested that the existence of various phenotypical differences in *C. sativus* species 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 confirmed by molecular analysis using right set of molecular markers. This is because the existence of molecular diversity depends upon the molecular markers used as well as amount of diversity existing among population used for the analysis (Namayandeh et al. 2013). The study of genetic diversity can provide an important piece of information for the strategic management and conservation of genetic resources and biodiversity at large (Odong et al. 2011). Therefore, the DNA fingerprint variation using RAPD and ISSR markers has revealed that there exists a rea-

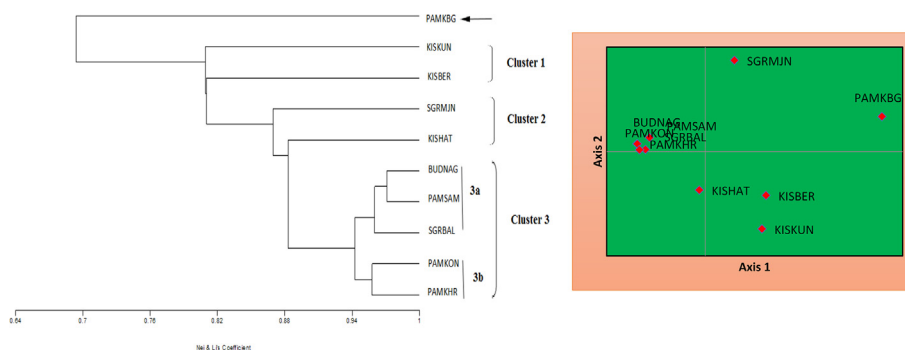


Fig. 4. A) Dendrogram illustrating genetic relationship among different samples of *Crocus sativus* L. Kashmirianus using RAPD-ISSR UPGMA clustering method. B) Two-dimensional principal coordinate analysis of *Crocus sativus* accessions based on RAPD-ISSR data.

Table 5Analysis of molecular variance (AMOVA) showing the partitioning of genetic variation within and among populations of *Crocus sativus* based on RAPD-ISSR data.

Source of variation	df	SS	MS	Est. Var.	%	Φ PT
Among Pops	2	53.933	26.967	2.018	9%	0.089
Within Pops	7	144.967	20.710	20.710	91%	
Total	9	198.900		22.728	100%	

Where: df = degree of freedom, SS = sum of squares, MS = mean squares, Est. var. = estimate of variance, % = percentage of total variation, Φ PT = PhiPT.

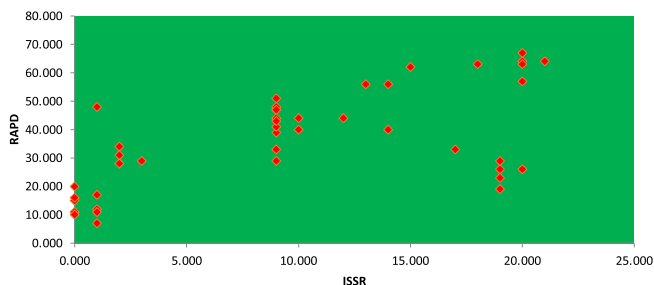


Fig. 5. Mantel test illustrating correlation between RAPD and ISSR markers ($r = 0.634$ and $P = 0.02$).

sonable genetic difference among *Crocus sativus* L. ‘Kashmirianus’ samples collected from Jammu and Kashmir. The existence of genetic variation among *Crocus sativus* could be because of appropriate climatic conditions for its growth and development (Larsen et al. 2015; Najafi Zarini et al. 2019; Nemati et al. 2014). The current research attempted to study molecular diversity of *Crocus sativus* L. as an effort to contribute information to the branch of saffronology about the existence of molecular diversity within saffron cultivars of Jammu and Kashmir (India) along with selection of appropriate molecular characterization markers. However, additional extensive and collaborative efforts are required to be initiated to study comparison of *Crocus sativus* L. “Kashmirianus” with *Crocus sativus* L. found elsewhere in the world using diverse number of molecular markers. The DNA from each polymorphic band could be eluted, cloned/sequenced for identification of sequence uniqueness and assigning the predicted functions using inter disciplinary approaches including functional genomics, bioinformatics etc. It is expected that through this approach, the global hunt to identify specific primers/markers could be achieved in order to break enigmatic nature of genetic diversity among *C. sativus* accessions.

5. Conclusion

Elucidating the genetic diversity analysis of *C. sativus* is an essential step to gain the global insights into existence of any probable polymorphism. In the present investigation, Nei-Li’s similarity index ranged between 0.70 and 0.97 along with Φ PT found as 0.089 which demonstrates existence of genetic diversity though limited. The polymorphic bands so obtained in the present study could be further exploited to decipher the nature of polymorphism among different cultivars of *C. sativus*. Therefore, it is very important to study comprehensively *Crocus sativus* diversity from different regions of the world and then follow the sequencing approach appropriately.

6. Ethics approval

Not Applicable.

7. Consent to participate

All authors consent to participate in this manuscript.

8. Consent for publication

All authors consent to publish this manuscript in Saudi journal of Biological Science.

9. Availability of data and material

Data will be available on request to corresponding or first author.

10. Code availability

Not Applicable.

11. Authors’ contributions

Mudasir A. Mir, Sheikh Mansoor, M. Sugapriya drafted and performed the experiments. Tabasum Ara, Mohammed Nasser Alyemeni and Leonard Wijaya analyzed the data. Mudasar A. Mir wrote the first draft of the manuscript. All authors read and approve the same for publication.

CRedit authorship contribution statement

Mudasir A. Mir: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft. **Sheikh Mansoor:** Conceptualization, Validation, Formal analysis, Writing - original draft. **M. Sugapriya:** Resources, Data curation, Writing - original draft. **Mohammed Nasser Alyemeni:** Software, Data curation, Writing - review & editing, Funding acquisition. **Leonard Wijaya:** Software, Formal analysis. **Parvaiz Ahmad:** Conceptualization, Validation, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sjbs.2020.11.063>.

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Further Reading

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