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Molecular Characterization of Saffron-Potential Candidates for Crop Improvement

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Abstract

In this study, thirty one (31) morphologically distinct selections of saffron crop were used for molecular characterization. Molecular characterization was done through SSR, ISSR and RAPD markers. RAPD and ISSR markers showed significant variation; however, SSR markers did not reveal any variation between the selected clones. The Jaccard's similarity coefficient ranged from 0.94 to 1.00 with an average of 0.98 among all 31 selections used. Minimum similarity value (0.94) was observed between CITH-S-107 and PAM-S-116 selections. The study provides sufficient knowledge to identify clones with better stigma characteristics for further crop improvement programs.

Keywords: *Crocus sativus,* genetic relationship, ISSR, molecular characterization, RAPD, SSR

Introduction

Crocus sativus L. is an autumn-flowering geophyte extensively grown in the Mediterranean basin and Near East since the Late Bronze Age (Negbi, 1999). Saffron, the dried red stigmas of *C. sativus*, has been used as flavouring and colouring agent since then and is currently considered the world's most expensive spice. The major components of saffron are the apocarotenoids cis- and trans-crocins, picrocrocin (β-D-glucopyranoside of hydroxyl-β-cyclocitral) and its degradation product, the odour-active safranal (Kanakis *et al*., 2004).

Many studies have demonstrated that the genotypic diversity of *C. sativus* is extremely low (Alavi Kia *et al*., 2008; Rubio-Moraga *et al*., 2009). This limited genetic diversity in saffron is attributed to its asexual propagation, followed by successive selection during breeding efforts (Alavi Kia *et al*., 2008). Most of the researchers reveal that saffron is monomorphic in nature by using RAPD, SSR and ISSR markers. But still some researchers (Qadri *et al*., 2012) believe that RAPD markers can be used for identifying the variation within these monomorphic genotypes. PCR-based approaches are in demand because of their simplicity and also because they have shown promise in crop improvement of a large number of crops. Genetic diversity and relationships among species or populations are important topics in genetics and plant breeding. Since saffron is generally monomorphic at morphological level, there is an urgent need to identify the variation at molecular level which can be further exploited for improvement of this crop. Even though qualitative traits of saffron are indeed influenced by sowing time (Gresta *et al*., 2008) and environmental conditions (Siracusa *et al*., 2012), only few and fragmentary information correlating genetic and biochemical traits is available to date. The discovery of genetic differences in saffron would mean a new way for its improvement, and eventually the possibility to link some particular genetic traits with morphological and biochemical features. Therefore, in order to explore the variability of morphological and qualitative traits among selected saffron clones, molecular evaluation, both at genomic and expression level, was done to identify saffron clones with higher variability in respect to improved quality.

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Material and methods

Plant material

Thirty one (31) different saffron clones representing the core collection for saffron germplasm conservation were used in the study. These clones were collected from Central Institute of Temperate Horticulture, Rangreth, Srinagar (J & K) and maintained at the Research Farm of said institution. Stigmas were collected from September to November 2011. Freshly cut stigmas were quickly immersed in liquid nitrogen and then stored at -80 °C for RNA isolation.

Molecular characterization

DNA extraction

DNA was extracted from 300 mg of leaf material using a modified Doyle and Doyle method (Doyle and Doyle, 1987). Leaf material was grounded to a fine powder in liquid nitrogen and placed in a microcentrifuge tube with 2 mL of extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA and 1.4 M NaCl) plus 40 µl of 2-mercaptoethanol. Following incubation at 65 °C for 30 min, 1.4 mL of chloroform:isoamyl

alcohol (24:1) was added, mixed and centrifuged at 8,000 rpm for 30 min; the supernatant was transferred to a new tube and then repeated three times. DNA was precipitated with isopropanol (2/3 volume of supernatant), then centrifuged at 8,000 rpm for 30 min, after which the supernatant was discarded and the pellet washed in 70% ethanol containing 10 mM ammonium acetate, for 20 min. The pellet was dissolved in 100 µL of TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA) and the DNA was re-precipitated with 1/2 volume of ammonium acetate 3 M and 2.5 volumes of ethanol. After centrifuging at 8,000 rpm for 30 min, the pellet was re-dissolved in TE buffer. The extracted DNA was quantified with a spectrophotometer and diluted to 30 ng/µL in TE. The DNA was stored at -20 °C for further analyses.

RAPD analysis

Samples were screened for RAPD variation using standard 10-base 128 primers specially developed for RAPD analysis, at constant low annealing temperature (34–37 °C). The PCR reaction (25 µl) contained the following: 1x reaction buffer (20 mM Tris-Cl pH 8.0, 50 mM KCl), 0.2 mM dNTPs, 2 mM $MgCl_2$, 10 Pm primer, 1.0 Unit of *Taq* DNA polymerase and 25 - 50 ng genomic DNA. The DNA was amplified in a thermal cycler that was programmed as follows: initial DNA denaturation for 5 min at 94°C; 45 cycles of 60 sec at 94°C (denaturation), 60 sec at 37 °C (annealing) and 120 sec at 72 °C (extension); followed by a final extension at 72°C for 7 min. All primers tested on all cultivars and markers were checked three times for reproducibility. The RAPD amplified-DNA was analyzed by electrophoresis on 2% agarose gel in a 0.5 x TBE buffer. The gels were stained with ethidium bromide (0.5 μgml^{-1}) and visualized under UV light.

ISSR and microsatellite analyses

DNA from individual plant of each saffron accession was screened with 31 pairs of SSR and 50 ISSR primers. The PCR reaction (25 µl) contained the following: 1 x reaction buffer (20 mM Tris- Cl pH 8.0, 50 mM KCl), 0.2 mM dNTPs, 2 mM $MgCl_2$, 10 pM primer, 1.0 Unit of *Taq* DNA polymerase and 25-50 ng genomic DNA. For standardization of annealing temperatures of SSR primers, gradient PCR was carried out in a gradient thermal cycler. Initial denaturation at 94 °C for 5 min was followed by 35 cycles at 94 °C for 1 min, 48-60 °C for 1 min and 72 °C for 2 min. The final extension was carried out at 72 °C for 7 min. For ISSR amplification products were run on 2% agarose and for microsatellites amplification 4% super-fine agarose was used. The bands were then visualized under UV light and photographed.

Molecular data analysis

The prominent DNA bands that were amplified by a given primer were scored as present (1) or absent (0) for all of the samples that were studied. In order to determine the utility of these markers, number of amplicons per primers, percent polymorphism, polymorphic information content (PIC), effective multiplex ratio (EMR)/resolving power (Rp) and marker index (MI) were calculated:

- Percent polymorphism was calculated as percentage of polymorphic loci from total loci obtained per primer.

- The polymorphism information content (PIC) value of individual primers were calculated based on the formula PIC = $2 \times F (1- F)$.

- Marker index, a product of information content, as measured by PIC, and effective multiplex ratio (EMR), the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay, was calculated following Powell *et al.* (1996) and resolving power (RP) of each primer combination was calculated according to Prevost and Wilkinson (1999).

The Jaccard's similarity index was calculated using NTSYS-pc version 2.02e package to compute pairwise Jaccard's similarity coefficients (Jaccard, 1908) and this similarity matrix was used in cluster analysis using an unweighted pair-group method with arithmetic averages (UPGMA) and sequential, agglomerative, hierarchical and nested (SAHN) clustering algorithm to obtain a dendrogram.

To judge the confidence of the group revealed in the dendrogram, bootstrap analysis was performed using the WINBOOT program with 1,000 replications.

The correlation of matrices obtained from SSR, ISSR and RAPD profiles was judged by two-way Mantel test (Mantel, 1967) using MxComp Module of NTSYSPC version 2.02e.

Results and discussions

Applications of molecular markers have opened up new insights on taxonomic analysis (Grilli-Caiola *et al*., 2004) in saffron. There are few articles that used molecular and biochemical approaches in an attempt to classify and clarify the systematic and phylogeny of this genus (Alavi-Kia *et al*., 2008; Frizzi *et al*., 2007; Grilli-Caiola *et al*., 2004; Keify and Beiki, 2012; Rubio-Moraga *et al*., 2009; Rubio-Moraga *et al*., 2010; Sik *et al*., 2008).

RAPD analysis

Amplified fragments from 128 RAPD primers varied in size from 200-1,500 bp. Fifteen primers were found polymorphic, with an average of 2.04 bands per primer. The respective values for overall genetic variability for PIC, Rp and MI across all the 31 clonal selections are given in Table 1. Highest PIC value (0.46) was observed in OPB1 and most of the primers showed zero value for PIC. Average PIC value was 0.03. The MI values ranged from 0.0 to 1.6 with an average of 0.06. Highest value (1.60) was scored with primers OPA14 and OPA17. RP ranged from 1.29 to 12.0 with an average of 3.97 per primer. Highest value (12.0) was scored with primers OPP6, OPV3 and OPA13. Allele number per locus varied from one to six (OPA13, OPP6, OPV3) and number of polymorphic bands range from 0 to 4 (OPA14, OPA17). Our study revealed that primer OPA14 shows 100% polymorphism by producing all four polymorphic bands, while primer OPA13, OPV3 and OPP6 produced the maximum number of bands, thus have a high marker index. Previously, in addition to morphological identification various biochemical and molecular markers have been used for genetic characterization of saffron genotypes (Keify and Beiki, 2012; Qadri *et al*., 2012). Moraga *et al*. (2009) found that RAPD markers could not distinguish the saffron genotypes at molecular level, while Qadri *et al*. (2012) found that RAPD markers have very good potential for studying genetic diversity in saffron. Caiola *et al*. (2004) found that amplification of seven *Crocus* species with 21 primers provided 217 repeatable and interpretable fragments, which are much higher than our findings. RAPD markers have been used for molecular association (Imran *et al*., 2008) and origin determination in saffron (Pardo *et al*., 2004).

Table 1. Polymorphic profile of 128 RAPD primers across 31 selections of saffron

Primer	NB	NPB	PIC	RP	MI	Primer	NB	NPB	PIC	RP	MI
OPA1	3	0	0.00	6.00	0.00	OPZ6	1	0	0.00	2.00	0.00
OPA2	2	0	0.00	4.00	0.00	OPZ7	2	0	0.00	4.00	0.00
OPA3	3	0	0.00	6.00	0.00	OPZ8	3	0	0.00	6.00	0.00
OPA4	4	0	0.00	8.00	0.00	OPZ9	2	0	0.00	4.00	0.00
OPA5	3	0	0.00	6.00	0.00	OPZ10	3	0	0.00	6.00	0.00
OPA7	3	0	0.00	6.00	0.00	OPP1	4	0	0.00	8.00	0.00
OPA11	5	0	0.00	10.00	0.00	OPP2	3	0	0.00	6.00	0.00
OPA12	2	0	0.00	4.00	0.00	OPP3	3	0	0.00	6.00	0.00
OPA13	6	0	0.00	12.00	0.00	OPP4	5	0	0.00	10.00	0.00
OPA14	4	4	0.40	5.16	1.60	OPP5	2	0	0.00	4.00	0.00
OPA15	1	1	0.35	1.55	0.35	OPP6	6	0	0.00	12.00	0.00
OPA16	2	2	0.24	3.41	0.66	OPP7	4	0	0.00	8.00	0.00
OPA17	4	4	0.40	5.16	1.60	OPP8	1	0	0.00	2.00	0.00
OPA20	2	2	0.20	1.61	0.41	OPP9	2	0	0.00	4.00	0.00
OPB1	1	1	0.46	1.29	0.46	OPP10	4	0	0.00	8.00	0.00
OPB2	1	0	0.00	2.00	0.00	OPO1	2	0	0.00	4.00	0.00
OPB3	1	0	0.00	2.00	0.00	OPO2	1	0	0.00	2.00	0.00
OPB4	2	0	0.00	4.00	0.00	OPO3	1	0	0.00	2.00	0.00
OPB5	1	0	0.00	2.00	0.00	OPO4	1	0	0.00	2.00	0.00
OPB6	1	0	0.00	2.00	0.00	OPO5	2	0	0.00	4.00	0.00
OPB7	1	0	0.00	2.00	0.00	OPO6	1	0	0.00	2.00	0.00
OPB8	1	0	0.00	2.00	0.00	OPO7	1	0	0.00	2.00	0.00
OPB9	1	0	0.00	2.00	0.00	OPO8	1	0	0.00	2.00	0.00
OPB10	1	0	0.00	2.00	0.00	OPO9	1	0	0.00	2.00	0.00
OPJ1	1	0	0.00	2.00	0.00	OPO10	1	0	0.00	2.00	0.00
OPJ2	2	0	0.00	4.00	0.00	OPS1	1	0	0.00	2.00	0.00
OPJ3	3	0	0.00	4.00	0.00	OPS2	1	0	0.00	2.00	0.00
OPJ4	1	0	0.00	2.00	0.00	OPS3	2	0	0.00	4.00	0.00
OPJ5	2	1	0.08	3.80	0.17	OPS4	2	0	0.00	4.00	0.00
OPJ6	2	2	0.09	3.80	0.18	OPS5	1	0	0.00	2.00	0.00
OPJ7	1	1	0.06	1.94	0.06	OPM1	2	0	0.00	4.00	0.00
OPJ8	1	1	0.06	1.94	0.06	OPM2	2	0	0.00	4.00	0.00
OPJ9	2	2	0.23	3.41	0.47	OPM3	1	0	0.00	2.00	0.00
OPJ10	3	0	0.00	6.00	0.00	OPM4	1	0	0.00	2.00	0.00
OPJ11	2	0	0.00	4.00	0.00	OPM5	2	0	0.00	4.00	0.00
OPJ12	3	0	0.00	6.00	0.00	OPL1	3	0	0.00	6.00	0.00
OPJ13	4	0	0.00	8.00	0.00	OPL2	2	0	0.00	4.00	0.00
OPJ14	3	0	0.00	6.00	0.00	OPL3	3	0	0.00	6.00	0.00
OPJ15	3	0	0.00	6.00	0.00	OPL4	4	0	0.00	8.00	0.00
OPV1	5	0	0.00	10.00	0.00	OPL5	3	0	0.00	6.00	0.00
OPV2	2	0	0.00	4.00	0.00	OPL6	3	0	0.00	6.00	0.00
OPV3	6	0	0.00	12.00	0.00	OPL7	2	0	0.00	4.00	0.00
OPV4	4	0	0.00	8.00	0.00	OPL8	2	0	0.00	4.00	0.00
OPV5	1	1	0.22	1.74	0.22	OPL9	2	0	0.00	4.00	0.00
OPV6	2	2	0.21	3.48	0.55	OPL10	2	0	0.00	4.00	0.00
OPV7	3	0	0.00	6.00	0.00	OPR1	1	0	0.00	2.00	0.00
OPV8	2	0	0.00	4.00	0.00	OPR2	2	2	0.24	3.41	0.66
OPV9	1	1	0.38	1.48	0.38	OPR3	4	0	0.00	8.00	0.00
OPV10	1	0	0.00	2.00	0.00	OPR4	2	0	0.00	4.00	0.00
OPU1	1	0	0.00	2.00	0.00	OPR5	1	0	0.00	2.00	0.00
OPU2	2	0	0.00	4.00	0.00	OPR5	1	0	0.00	2.00	0.00
OPU3	1	0	0.00	2.00	0.00	OPR6	1	0	0.00	4.00	0.00
OPU4	1	0	0.00	2.00	0.00	OPR7	2	0	0.00	2.00	0.00
OPU5	1	0	0.00	2.00	0.00	OPR8	1	0	0.00	2.00	0.00
OPU6	1	0	0.00	2.00	0.00	OPR9	1	0	0.00	2.00	0.00
OPU7	1	0	0.00	2.00	0.00	OPR10	1	0	0.00	2.00	0.00
OPU8	1	0	0.00	2.00	0.00	OPT1	1	0	0.00	2.00	0.00
OPU9	1	0	0.00	2.00	0.00	OPT2	1	0	0.00	2.00	0.00
OPU10	2	0	0.00	4.00	0.00	OPT4	1	0	0.00	2.00	0.00
OPZ1	2	0	0.00	4.00	0.00	OPT5	2	0	0.00	4.00	0.00
OPZ2	1	0	0.00	2.00	0.00	OPC1	2	0	0.00	4.00	0.00
OPZ3	2	0	0.00	4.00	0.00	OPC2	1	0	0.00	2.00	0.00
OPZ4	2	0	0.00	4.00	0.00	OPC3	2	0	0.00	4.00	0.00
OPZ5	1	0	0.00	2.00	0.00	OPC4	2	0	0.00	4.00	0.00

NB- Number of bands; NBP- Number of polymorphic bands; PIC- Polymorphic information content; RP- Resolving power; MI- Marker index

SSR analysis

Thirty one SSR primers resulted in number of amplified fragments which varied in size from 160-400 bp. None of the primers was found polymorphic. Bands per primer range from 1 to 2, with an average of 1.1 bands per primer. Since all the primers were found monomorphic across the 31 selections, thus all SSR primers show zero values for PIC and MI. Rp value range from 2-4 (CSMIC19, CSMIC43 and CSMIC51). Allele number per locus varied from 1 to 2 (CSMIC19, CSMIC43 and CSMIC51) with an average of 1.1 alleles per loci (Table 2), which is very low. Our findings show that SSR primers, although considered very good markers with high reproducibility, could not identify any polymorphism among the selected clones. Hence, there is need to develop more SSR markers for scanning more of the saffron genome. Moraga *et al.* (2009) also found that existing SSR markers could not distinguish saffron genotypes. Microsatellite markers are useful for genetic diversity analysis studies at varietal, species and genus level, due to their sequence conservation at flanking regions (Hamza *et al*., 2004). Also there is need to exploit functional genomic variation in saffron so that EST-SSR markers could be developed for identifying and exploiting variation in these saffron selection. The major constraint of using SSR markers from genomic libraries is the high development cost and the effort required to obtain working primers for a given study species. This has restricted their use to only a few of the agriculturally important crops. Recently, a new alternative source of SSRs development from genomic and expressed sequence tag (EST) databases has been utilized (Ozkan *et al*., 2013). With the availability of a large number of ESTs and other DNA sequence data, development of EST-based SSR markers through data mining has become a fast, efficient and relatively inexpensive method compared with the development of genomic SSRs (Gupta *et al*., 2003).

ISSR analysis

Amplified fragments from 50 ISSR primers varied in size from 150–900 bp. Only five out of fifteen primers were found polymorphic, with an average of 1.06 bands per primer. The respective values for overall genetic variability for PIC, Rp and MI across all the 31 clonal selections are given in Table 3. Highest PIC value (0.32) was observed for ISCS8 and ISCS24. The average PIC value was 0.018. The MI values ranged from 0.0 to 0.65 with an average of 0.04. Highest value (0.65) was scored with primers ISCS8 and ISCS24. RP ranged from 1.93 to 3.81, with an average of 2.07 per primer. Highest value (3.81) was scored with the primer ISCS1 and the lowest value (1.94) for the primers ISCS12 and ISCS18. Allele number per locus varied from 1 to 2 (ISCS1, ISCS8, ISCS24) and number of polymorphic bands range from 0 to 2 (ISCS8 and ISCS24). Hence our findings suggest that ISSR markers ISCS1, ISCS8, ISCS24 have desirable values for all primer parameters (PIC, Rp and MI), therefore they can have potential for studying genetic diversity in saffron. Our study revealed that ISSR markers can be used for studying the genetic diversity in saffron, but should be used along with other markers and more ISSR primers with higher number of selections. Moraga *et al.* (2009) found that ISSR markers could not reveal any polymorphism in saffron. ISSRs exhibit the specificity of

Table 2. Genetic diversity profile of 31 SSR primers across 31 selections of saffron

Primer	Forward Primer sequence 5'-3'	Reverse Primer sequence 5'-3'	Amplicon size bp	PIC	RP	MI
CSMIC7	GAATCACCACCTGAATTGTGAG	AAGAGGTCGAAGAAGGGAAAAC	280	0.0	2.0	0.0
CSMIC8	TCTTGGAATGGTTAGAGCGTGT	ACCAGCAATCTTTGGAACAGTC	300	0.0	2.0	0.0
CSMIC9	ACTGAAAGAAAGGGGAGAAAGG	TATATCGAATGGAGGTTCCGTC	230	0.0	2.0	0.0
CSMIC10	AATCACACACAACATGGTCGTT	TGTTTAACCCAGCTAGCAGAAT	300	0.0	2.0	0.0
CSMIC19	GGCCTAGCTAGCAGAATCACAA	AGCTAGCAGAATCACACTCTT	300 & 400	0.0	4.0	0.0
CSMIC21	CTCGCTAGCCGAATCACAACT	TGTGAGAGCACACGGTGT	200	0.0	2.0	0.0
CSMIC26	ATCACTCATAACTCTCCATGA	AGCTAGCAGATCACATAGGT	350	0.0	2.0	0.0
CSMIC27	TGGATATACGTAATCCAGACT	ATAATTCCTGAGGTGAATT	300	0.0	2.0	0.0
CSMIC28	TAAGGCCATGCTAGCAGAAT	AGCAGTAATTCGTAGCGACA	310	0.0	2.0	0.0
CSMIC30	GCAGAATCACACATGGTTACAA	AGTGACACGACACTCACTAT	260	0.0	2.0	0.0
CSMIC38	GTCTAAGGCCTAGCTAGCAGA	GTTATCAAATGTTGGCCCACT	300	0.0	2.0	0.0
CSMIC39	GCTAGCAGAATCACTACTTGA	AATGTTGGCCCACTCACACT	350	0.0	2.0	0.0
CSMIC43	GCAGAATCACTACTTGAAGACA	TGAGATGGATATATTCTCTGA	250 & 350	0.0	4.0	0.0
CSMIC544	CAGTGCTTCGGCTGAATGTGAA	ACTGCTGGACGGTGCAACTT	200	0.0	2.0	0.0
CSMIC45	CCGCCTAGCTAGCAGAATCACA	GATAAGACCTGCAACTTCAACT	160	0.0	2.0	0.0
CSMIC46	GTACAGTGCTGAAGAGGAGGA	TGGATACGCTGCACGTATCTCA	220	0.0	2.0	0.0
CSMIC47	ACCAGGTCAGTTGATGCCTCAT	CAGTGTAGCTACTTAGACAGT	250	0.0	2.0	0.0
CSMIC48	GCGAGCGAAATCACAATCTCGA	GCGAGCGAAATCACAATCTCGA	250	0.0	2.0	0.0
CSMIC49	ACTAGTTCACTCATCCGTTA	TGAATCGAATGGGTAGGGAAT	200	0.0	2.0	0.0
CSMIC50	TAACCTCGTCGGAGCGGTGGA	GGAGCAACAATGGCGGTGGAA	240	0.0	2.0	0.0
CSMIC51	GACGGGTAGTAGAAAGTTCTTCA	CGAATGGGTCTCCAAACCCT	250 & 300	0.0	4.0	0.0
CSMIC53	GCAGAATCACTGCTGGACGGGT	CAGTGCTTCGGCTGAATGTGAA	220	0.0	2.0	0.0
CSMIC54	AGCAGCAGAGAAGTAAGACAGT	TCAACTTCCCAACCACTTTGA	160	0.0	2.0	0.0
CSMIC55	AGCAACAGAGGCACACATTCA	AGCTGTCAGTCCAATCATCAAC	270	0.0	2.0	0.0
CSMIC56	CTTATTGGATACGCTGCA	TAAGCCTAGCTAGCAGAA	260	0.0	2.0	0.0
CSMIC57	GTAACCTGCTCCAGTGCTA	TAAGGCCTAGCTAGCAGAATCT	320	0.0	2.0	0.0
CSMIC58	TCGTCATGGTCGTCGCTACTA	AGCCTAGCTAGCAGATCATAGA	260	0.0	2.0	0.0
CSMIC59	GAATATTGTTGATGAGGCCGGA	AAGAGAGATATTAAATAAGTCGCA	200	0.0	2.0	0.0
CSMIC60	CATCGGCCTGAATGCCGT	GGGAAGTTCAAATCCCACTA	200	0.0	2.0	0.0
CSMIC61	TTCAAGTGCTTATTGGTCCA	CATGTTCAATGCTTCATCAAGT	318	0.0	2.0	0.0
CSMIC62	CCAATCTGAGGACGGGCT	AGAAGCGTGATGAAGTGA	350	0.0	2.0	0.0

NB- Number of bands; NBP- Number of polymorphic bands; PIC- Polymorphic information content; RP- Resolving power; MI- Marker index

microsatellite markers, but need no sequence information for primer synthesis enjoying the advantage of random markers (Joshi *et al*., 2000). ISSR markers usually show high polymorphism (Kojima *et al*., 1998) although the level of polymorphism has been shown to vary with the detection method used. Like RAPDs, reproducibility, dominant inheritance and homology of co-migrating amplification products are the main limitations of ISSRs.

Two-way Mantel test (Mantel, 1967) was done between the ISSR and RAPD data matrices. The correlation coefficient was estimated to be 0.33 between the matrices generated by ISSR and RAPD markers using the Mantel Test (t 2.43, P 0.99). Since SSR markers produce invariant similarity matrix due to monomorphic data hence could not be used for determining correlation through Mantel test.

Genetic relationship

In order to find out the genetic relationship between different saffron selections, SSR, ISSR and RAPD data sets were combined together and this general view data was used for analysis using NTSYS$_{PC}$ version 2.02e. The Jaccard's similarity coefficient ranged from 0.94 to 1.00 with an average of 0.98 among all the 31 selections used (Table 4). Minimum similarity value (0.94) was observed between CITH-S-107 and PAM-S-116 selections. The genetic relationship between the accessions was clearly depicted in the dendrogram which was

Table 3. Polymorphic profile of 50 ISSR primers across 31 selections of saffron

Primer	Primer Sequence 5'-3'	NB	NPB	PIC	RP	MI
ISCS1	TCCTCCTCCTCCTCCTCCC	2	1	0.17	3.81	0.35
ISCS2	GATGATGATGATGATGATG	1	0	0.00	2.00	0.00
ISCS3	CTTCTTCTTCTTCTTCTTCTTC	1	0	0.00	2.00	0.00
ISCS4	TGGTGGTGGTGGTGGTGGC	1	0	0.00	2.00	0.00
ISCS5	GTGTGTGTGTGTGTGTG	1	0	0.00	2.00	0.00
ISCS6	GAGAGAGAGAGAGAGAG	1	0	0.00	2.00	0.00
ISCS7	TCTCTCTCTCTCTCTCTCC	1	0	0.00	2.00	0.00
ISCS8	TCTTCTTCTTCTTCTTCTTCTTCTTCTA	2	2	0.32	3.03	0.65
ISCS9	ATCATCATCATCATCATCATCATCATCATCG	1	0	0.00	2.00	0.00
ISCS10	ACACACACACACACC	1	0	0.00	2.00	0.00
ISCS11	CTCTCTCTCTCTCTCTT	1	0	0.00	2.00	0.00
ISCS12	TTGTTGTTGTTGTTGTTGC	1	1	0.06	1.94	0.06
ISCS13	TATTATTATTATTATTATTATTATG	1	0	0.00	2.00	0.00
ISCS14	AGTGAGTGAGTGAGTGAGTGA	1	0	0.00	2.00	0.00
ISCS15	TATCTATCTATCTATCTATCTATCT	1	0	0.00	2.00	0.00
ISCS16	HBHGAGGAGGAGGAGGAG	1	0	0.00	2.00	0.00
ISCS17	DBDBCACCACCACCACCAC	1	0	0.00	2.00	0.00
ISCS18	DBDBCCACCACCACCACCA	1	1	0.06	1.94	0.06
ISCS19	HVHGTGGTGGTGGTGGTG	1	0	0.00	2.00	0.00
ISCS20	DHBCGACGACGACGACGA	1	0	0.00	2.00	0.00
ISCS21	BDBACAACAACAACAACA	1	0	0.00	2.00	0.00
ISCS22	HBBGAAGAAGAAGAAGAA	1	0	0.00	2.00	0.00
ISCS23	HBDBGACCGACCGACCGACC	1	0	0.00	2.00	0.00
ISCS24	HBVBGATAGATAGATAGATA	2	2	0.32	3.03	0.65
ISCS25	HBVCGATCGATCGATCGAT	1	0	0.00	2.00	0.00
ISCS26	GTGTGTGTGTGTGTGTYG	1	0	0.00	2.00	0.00
ISCS27	TCTCTCTCTCTCTCTCRA	1	0	0.00	2.00	0.00
ISCS28	TCTCTCTCTCTCTCTCRT	1	0	0.00	2.00	0.00
ISCS29	TCTCTCTCTCTCTCTCRG	1	0	0.00	2.00	0.00
ISCS30	ACACACACACACACACYT	1	0	0.00	2.00	0.00
ISCS31	ACACACACACACACACYA	1	0	0.00	2.00	0.00
ISCS32	ACACACACACACACACYG	1	0	0.00	2.00	0.00
ISCS33	TGTGTGTGTGTGTGTGRT	1	0	0.00	2.00	0.00
ISCS34	TGTGTGTGTGTGTGTGRC	1	0	0.00	2.00	0.00
ISCS35	TGTGTGTGTGTGTGTGRA	1	0	0.00	2.00	0.00
ISCS36	ACCACCACCACCACCACC	1	0	0.00	2.00	0.00
ISCS37	AGCAGCAGCAGCAGCAGC	1	0	0.00	2.00	0.00
ISCS38	GTGTGTGTGTGTGTGTYC	1	0	0.00	2.00	0.00
ISCS39	ATGATGATGATGATGATG	1	0	0.00	2.00	0.00
ISCS40	CCGCCGCCGCCGCCGCCG	1	0	0.00	2.00	0.00
ISCS41	CTCCTCCTCCTCCTCCTC	1	0	0.00	2.00	0.00
ISCS42	GGCGGCGGCGGCGGCGGC	1	0	0.00	2.00	0.00
ISCS43	GAAGAAGAAGAAGAAGAA	1	0	0.00	2.00	0.00
ISCS44	GTTGTTGTTGTTGTTGTT	1	0	0.00	2.00	0.00
ISCS45	TGCTGCTGCTGCTGCTGC	1	0	0.00	2.00	0.00
ISCS46	GTGTGTGTGTGTGTGTYA	1	0	0.00	2.00	0.00
ISCS47	CACACACACACACACARG	1	0	0.00	2.00	0.00
ISCS48	GACAGACAGACAGACA	1	0	0.00	2.00	0.00
ISCS49	CCCTCCCTCCCTCCCT	1	0	0.00	2.00	0.00
ISCS50	CACACACACACACACARC	1	0	0.00	2.00	0.00

NB- Number of bands; NBP- Number of polymorphic bands; PIC- Polymorphic information content; RP- Resolving power; MI- Marker index

constructed from the DNA profile. The dendrogram showed that all the selections are closely related with very low variability (Fig. 1).

The genetic closeness among the selections can be explained by the high degree of commonness in these selections, due to lack of sexual reproduction and geographically closed locations. All the 31 selections formed five main clusters with some degree of sub-clustering within. Cluster I, II, III, IV and V represent four selections (CITH-S-125, CITH-S-115, PAM-S-13 and PAM-S-101), 15 (CITH-S-124, PAM-S-3, PAM-S-106, CITH-S-122, CITH-S-114, PAM-S-102, PAM-S-111, BUS-S-76, CITH-S-12, CITH-S-113, CITH-S-112, CITH-S-105, PAM-S-11, PAM-S-108 and CITH-S-103), 5 (CITH-S-121, CITH-S-118, CITH-S-120, CITH-S-104 and CITH-S-117), 3 (BUD-S-110, CITH-S-10 and CITH-S-119) and 3 selections (CITH-S-123, CITH-S-43 and CITH-S-107) respectively. Cluster I shows average similarity of 98.7%, 98%, 97.8% and 98% with cluster II, III, IV and V respectively. However, within the cluster I the average similarity was 99.4% and within cluster II, III, IV and V it was 99.0%, 98.7%, 98% and 98.6%, respectively. Cluster II shows average similarity coefficient of 98.4% with cluster III, IV and V; Cluster III shows 97.8% and 97.9% of average similarity with cluster IV and V respectively, and cluster IV shows 97.3% similarity with cluster V. Never the less, the minimum similarity (97.3%) observed between clusters IV and V is very high; this revealed that the selections are closely related at molecular level. Selection PAM-S-116 did not form part of any cluster and form separate co-ordinate in PCO analysis. The principal coordinate analysis (PCO) showed that the first three axes accounted for 98.5% (97.7, 0.4 and 0.32 by Ist, 2nd and 3rd co-ordinate respectively) of total variation. The grouping shown in dendrogram was at par with that shown in 3D scatter (Fig. 2). The results obtained using 128 RAPD, 50 ISSR and 31 SSR primers that yield a total of 34 polymorphic loci, produced a fingerprint that could be used for diversity analysis and identification of diverse selections among the selected ones (Fig. 3). Also, association of molecular marker data can be done with morphological parameters. Very little study has been done on association of molecular data with morphological or yield related characters. Studies in relation to genetic variability and divergence in saffron have been studied (Anonymous, 2006). Genetic variation and heritability of agro-morphological and photochemical traits in Iranian saffron populations have been studied, and populations were found significantly different for most evaluated traits, like leaf number per plant, leaf length, flower number per plot, dry stigma weight per plot, spathe number and the content of crocins, picrocrocin and safranal (Baghalian *et al*., 2010). Moraga *et al*. (2009) found that saffron is a monomorphic species as revealed by RAPD, ISSR and microsatellite analyses. Pardo *et al*. (2004) investigated the distinction and variability of *Crocus sativus* from several geographic areas (Italy, Iran, Greece and Spain) using molecular markers and dry stigmas as plant material. Zubor *et al*. (2004) used AFLP markers to study the genetic diversity among different saffron species and found the close relationship between these species. AFLP has been found an effective tool for identifying genetic variability in saffron (*Crocus sativus* L.) of different origin (Siracusa *et al*., 2012). Reterotransposons have also been used for studying the genetic diversity among different saffron species, and genetic variation was observed within and between species, whereas in some cases variation was found among ecotypes of the same species from different geographical regions (Alavi-Kia *et al*., 2008). Use of molecular markers as a tool for identification of variability among different saffron clones is an important area for improvement of this crop through breeding. Sequencing of corm cDNAs at different developmental stages would increase our knowledge about the physiological processes occurring in this organ. Little work has been done so for in these areas, in regard to the development of gene expression in saffron corm at different time intervals (Ortí *et al*., 2004). Different ESTs with respect to corm development, signal perception and transduction, defense against pathogen and stress, metabolism, development and gene regulation, cell organization, protein metabolism, transport etc. have been identified. EST data-base from saffron stigmas has been produced (D'Agostino *et al*., 2007), and this will be very useful for detecting the level of expression of different components in saffron genotypes. Furthermore, this data-base will be very useful for designing functional markers (EST-SSR) which can be used for identifying the variation among different saffron selections at transcriptional level. Since present markers did not reveal any significant polymorphism at

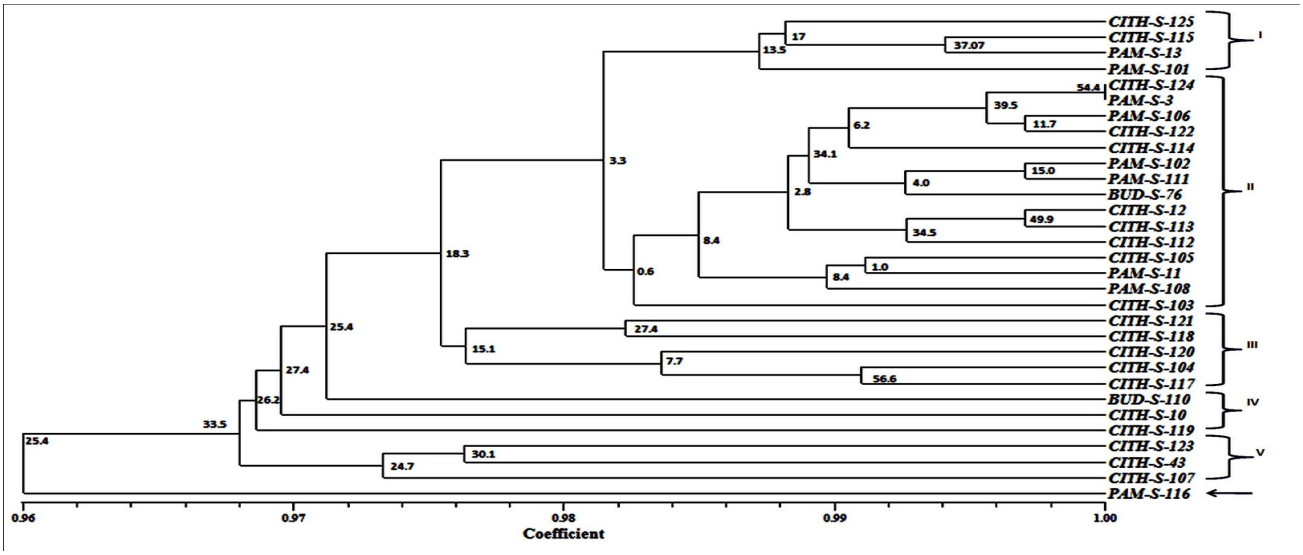

Fig. 1. UPGMA dendrogram showing clustering pattern of saffron selections. The bootstrap values are given on the nodes

Table 4. Jaccard's similarity matrix between 31 saffron genotypes

	CITH-S 125	CITH-S 123	CITH-S 124	PAM-S 116	CITH-S 12	CITH-S 121	CITH-S 120	CITH-S 107	CITH-S 104	CITH-S 117	CITH-S 112	CITH-S 113	CITH-S 119	CITH-S 118	CITH-S 10	CITH-S 103	CITH-S 43	CITH-S 114	CITH-S 115	CITH-S 105	PAM-S 106	PAM-S 102	PAM-S 108	PAM-S 11	CITH-S 122	PAM-S 13	PAM-S 101	PAM-S 3	PAM-S 111	BUD-S 110	BUD-S 76
CITH-S 125	1.00																													
CITH-S 123	0.97	1.00																												
CITH-S 124	0.98	0.98	1.00																											
PAM-S 116	0.96	0.95	0.97	1.00																										
CITH-S 12	0.98	0.97	0.99	0.97	1.00																									
CITH-S 121	0.97	0.97	0.98	0.95	0.98	1.00																								
CITH-S 120	0.97	0.97	0.98	0.95	0.98	0.98	1.00																							
CITH-S 107	0.95	0.97	0.97	0.94	0.97	0.97	0.97	1.00																						
CITH-S 104	0.97	0.96	0.97	0.95	0.98	0.97	0.98	0.98	1.00																					
CITH-S 117	0.97	0.95	0.97	0.95	0.98	0.97	0.99	0.98	0.99	1.00																				
CITH-S 112	0.97	0.97	0.99	0.97	0.99	0.98	0.98	0.97	0.97	0.98	1.00																			
CITH-S 113	0.98	0.97	0.99	0.97	1.00	0.98	0.98	0.97	0.98	0.98	0.99	1.00																		
CITH-S 119	0.97	0.95	0.97	0.95	0.98	0.97	0.96	0.97	0.98	0.97	0.97	0.98	1.00																	
CITH-S 118	0.97	0.96	0.98	0.95	0.98	0.98	0.98	0.98	0.98	0.99	0.98	0.98	0.98	1.00																
CITH-S 10	0.96	0.96	0.98	0.97	0.98	0.97	0.97	0.96	0.96	0.96	0.98	0.98	0.96	0.97	1.00															
CITH-S 103	0.97	0.97	0.99	0.96	0.98	0.98	0.98	0.97	0.97	0.97	0.98	0.98	0.97	0.98	0.97	1.00														
CITH-S 43	0.97	0.98	0.97	0.95	0.97	0.97	0.95	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.95	0.96	1.00													
CITH-S 114	0.98	0.98	0.99	0.96	0.99	0.98	0.98	0.97	0.97	0.96	0.99	0.98	0.97	0.98	0.97	0.99	0.97	1.00												
CITH-S 115	0.99	0.97	0.98	0.96	0.99	0.97	0.98	0.96	0.98	0.97	0.99	0.98	0.97	0.97	0.96	0.97	0.98	0.98	1.00											
CITH-S 105	0.98	0.98	0.99	0.96	0.98	0.99	0.98	0.96	0.97	0.96	0.98	0.98	0.97	0.98	0.97	0.99	0.97	0.99	0.98	1.00										
PAM-S 106	0.98	0.98	0.99	0.97	0.99	0.98	0.98	0.97	0.98	0.97	0.99	0.99	0.97	0.98	0.97	0.99	0.97	0.99	0.98	0.99	1.00									
PAM-S 102	0.98	0.98	0.99	0.97	0.99	0.97	0.98	0.97	0.98	0.97	0.99	0.99	0.97	0.97	0.97	0.98	0.97	0.99	0.99	0.99	1.00	1.00								
PAM-S 108	0.98	0.97	0.99	0.96	0.98	0.98	0.99	0.96	0.98	0.97	0.98	0.98	0.97	0.98	0.97	0.99	0.96	0.99	0.98	0.99	0.99	0.99	1.00							
PAM-S 11	0.98	0.98	0.99	0.96	0.98	0.98	0.99	0.97	0.97	0.97	0.98	0.98	0.96	0.97	0.97	0.98	0.97	0.99	0.99	0.99	0.99	0.99	0.99	1.00						
CITH-S 122	0.98	0.98	1.00	0.97	0.99	0.98	0.98	0.97	0.97	0.97	0.99	0.99	0.97	0.98	0.98	0.98	0.97	0.99	0.99	0.99	1.00	0.99	0.99	0.99	1.00					
PAM-S 13	0.99	0.97	0.99	0.97	0.99	0.97	0.98	0.96	0.98	0.98	0.99	0.99	0.97	0.97	0.97	0.97	0.97	0.98	0.99	0.98	0.99	0.99	0.99	0.99	0.99	1.00				
PAM-S 101	0.99	0.97	0.98	0.97	0.98	0.96	0.97	0.95	0.97	0.97	0.98	0.98	0.96	0.96	0.96	0.98	0.96	0.97	0.99	0.97	0.98	0.98	0.98	0.98	0.98	0.99	1.00			
PAM-S 3	0.98	0.98	1.00	0.97	0.99	0.98	0.98	0.97	0.97	0.97	0.99	0.99	0.97	0.98	0.98	0.99	0.97	0.99	0.98	0.99	0.99	0.99	0.99	0.99	1.00	0.99	0.98	1.00		
PAM-S 111	0.98	0.98	0.99	0.97	0.99	0.97	0.98	0.97	0.98	0.97	0.99	0.99	0.97	0.97	0.97	0.98	0.97	0.99	0.99	0.98	0.99	1.00	0.99	0.99	0.99	0.99	0.99	0.99	1.00	
BUD-S 110	0.96	0.97	0.98	0.97	0.97	0.97	0.97	0.96	0.97	0.96	0.97	0.97	0.95	0.96	0.97	0.97	0.96	0.98	0.97	0.97	0.98	0.98	0.97	0.97	0.98	0.97	0.96	0.98	0.98	1.00
BUD-S 76	0.98	0.98	0.98	0.97	0.99	0.97	0.98	0.97	0.98	0.97	0.98	0.98	0.97	0.97	0.96	0.97	0.97	0.98	0.98	0.98	0.99	0.99	0.98	0.99	0.99	0.99	0.99	0.98	0.99	0.98	1.00

genetic level between different saffron selections, there is the need to develop more number of markers, particularity SSR markers, which can be used for high density profiling. Sequencing of saffron genome can also play an important role in designing different types of quality molecular markers. But no serious efforts have been made in sequencing saffron genome yet.

Our study revealed that selections chosen for this study showed variation at molecular level, which can further be deciphered by using more number of molecular markers for high throughput scanning and high density profiling of saffron genome. Further study needs to be done to identify divergent selections with respect to different traits for improvement of this crop.

Conclusions

To slow down or stop geographical decline in saffron cultivation and, rather, to achieve an appreciable expansion of saffron-producing areas, a substantial increase in productivity by selecting superior clones is the need of the hour. The creation of new cultivars based on the identification of clones with higher apocarotenoid biosynthetic potential will facilitate this process. There is an urgent necessity to study the genetic and selection potential of various populations of saffron in Kashmir, as well as the populations in each region separately. Present study screened 31 morphologically distinct saffron clones and identified clones with better quality stigma, higher yield and maximum apocarotenoid biosynthetic potential. Although our study revealed that these clones selected are not divergent at molecular level, however, these can be mass multiplied under Kashmir conditions for saffron crop improvement and economic development.

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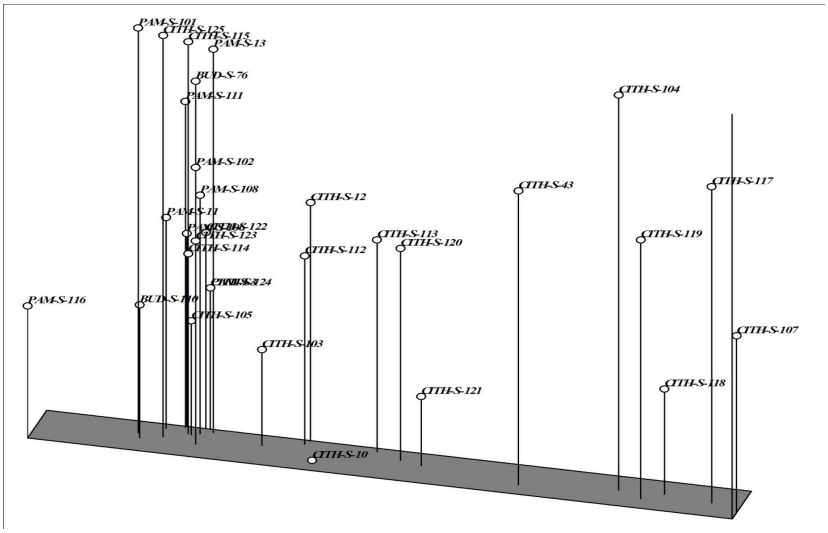

Fig. 2. Three dimensional PCO (principal co-ordinate analysis) scaling of 31 saffron genotypes using RAPD, ISSR and SSR markers

Fig. 3. Fingerprint derived from polymorphic markers representing level of polymorphism between 31 saffron selections (C: CITH; P: PAM and B: BUD)

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