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Original scientific paper

IDENTIFICATION OF MARKERS ASSOCIATED WITH TRAITS FOR USE IN MARKER-ASSISTED SELECTION IN SAFFRON

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Determination of association between molecular markers and agronomic traits provides an excellent tool for marker-assisted selection. In this study, multivariate stepwise regression analysis was used to estimate associations between SSR markers and some agronomic traits in saffron ecotypes. Two-year average values for the measured traits were used for association analyses. The results of stepwise regression analysis revealed significant associations between the traits and some of the studied loci. More than one informative marker was detected for most of the traits. Totally 25 informative SSR markers were identified in two years. Markers SCA382, SCA15 and SCD219 were associated with most traits under both years. These markers are considered to be relatively more reliable. Among the SSR primers, special attention should be drawn to primers SCA382, SCA15, and SCD219, which had the highest associated fragments with most traits in two years and could be considered for use as candidate markers in marker-assisted selection.

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INTRODUCTION

Saffron (*Crocus sativus* L.) is the oldest and most expensive of all aromatic, and medicinal plants (GHORBANI, 2006; TURHAN *et al.*, 2007). Saffron has been used since the ancient times (FERNÁNDEZ and PANDALAI, 2004) for different purposes: medicinal use (ABDULLAEV and ESPINOSA-AGUIRRE, 2004; CHRYSSANTHI *et al.*, 2009; DALEZIS *et al.*, 2009; MAGESH *et al.*, 2006), as aromatic plant, food, for scientific purposes (ANASTASAKI *et al.*, 2010), textile dyeing, perfume extraction (FERNÁNDEZ and PANDALAI, 2004), etc. Saffron cultivation in the world, which shows a wide range of adaptability to soil types, temperatures and day length, encourage its production from the Mediterranean basin to Middle East (KAFI, 2006; MORAGA *et al.*, 2009). Iran is currently the largest saffron producer in the world, providing over 93.7% of its global supply (GHORBANI, 2007). Major saffron producers are the following countries: Iran, Spain, India, Greece and Morocco (JALALI-HERAVI *et al.*, 2010), Iran being the world's most important producer (JALALI-HERAVI *et al.*, 2010; KUMAR *et al.*, 2008).

The development of morphological traits is occurring during plant growth stage. They may be limited in number and influenced by environmental factors or the developmental stage of the plant (STUBER *et al.*, 1999). These marker types have been superseded by DNA-based methods generating "fingerprints" which are distinctive patterns of DNA fragments typically subjected to high resolution gel electrophoresis and detected by staining or labeling (SCHULMAN, 2007). Since DNA markers show the variation at the DNA level, and are not affected by environmental conditions, they are more reliable than morphological markers (16, 24). One of the favorite marker systems has been SSR (simple sequence repeat or microsatellite) marker. This type of marker offers a number of advantages over other marker systems: multiple allelic, co-dominants, locus specificity, interspersed throughout the genome and high polymorphism. All these attributes make microsatellites suitable for use in population genetic and diversity studies (BALDONI *et al.*, 2006; NEMATI *et al.*, 2012).

Determination of association between molecular markers and morphological traits provide an excellent tool for indirect selection of a trait of interest in the population. This has important applications to the study of relations between molecular markers and agronomic traits, some of which include: the detection and analysis of potential in specific genotypes, collections of germplasm, identification of desirable alleles, and validation of candidate markers linked to quantitative traits (GEBHARDT *et al.*, 2004). To overcome these limitations, multiple regression analysis offers an appropriate method to identify markers associated with the trait. Multiple regression analysis is a statistical process for estimating relationships among molecular markers as independent variables and morphological traits as dependent variables. It is the way to determine the coefficient of determination R^2; it gives the proportion of the variance (fluctuation) of dependent variable that can be predicted from the independent variable (GOMEZ and GOMEZ, 1984).

According to the study of KHADIVI-KHUB (2014) by multiple regression analysis, 33 SSR alleles and 135 RAPD fragments were found associated with 14 of affecting fruit traits. Some of SSR and RAPD markers were associated with more than one fruit trait in multiple regression analysis. MARSAFARI *et al.* (2014) investigated the association of 11 morphological traits with molecular markers in 15 cultivars of date palm. All regression models were significant for ISSR and RAPD marker and at the level of 1% for all traits. Of 294 DNA markers (162 ISSR markers

and 132 RAPD markers), 173 markers (89 ISSR markers and 84 RAPD markers) showed association, with at least one of 11 traits of fruit, stone and tree performance characteristics in both marker systems. BASAKI *et al.* (2011) reported that 14 traits (excluding fruit shape, calyx type, hull cracking sensitivity and skin color) showed significant association with 14 SSR bands. The association markers explained 2% to 29% of the variation of individual traits.

In this study, multiple regression analysis was used to identify associations between SSR markers with some agronomic traits in saffron ecotypes.

MATERIALS AND METHODS

Plant materials

In this study, 60 saffron accessions were collected from most ancient cultivation areas in Iran. Ten samples were collected from each region. The details of the accessions and their geographic origins are listed in *Tab. 1*.

Table 1- Sampling region, province and country of saffron ecotypes

No	Region	Province	Country	Elevation	Latitude	Longitude
1	Mashhad	Razavi Khorasan	Iran	985 m	36.26046 North	59.61675 East
2	Torbat-e Jam	Razavi Khorasan	Iran	904 m	35.23169 North	60.64012 East
3	Gonabad	Razavi Khorasan	Iran	1098 m	34.33955 North	58.70303 East
4	Torbat-e Heydariyeh	Razavi Khorasan	Iran	1330 m	35.27984 North	59.21614 East
5	Birjand	South Khorasan	Iran	1491 m	32.8649 North	59.22625 East
6	Ghayen	South Khorasan	Iran	1455 m	33.72267 North	59.17882 East

An average of fifty samples per plot were used to measure the traits of Fresh Stigma Weight (FSW), Dry Stigma Weight (DSW), Stigma Length (SL), Fresh Flower Weight (FFW) and Dry Flower Weight (DFW). Ten samples in each plot were used to evaluate the traits of Dry Stigma Yield (DSY), Flower Number (FN), Leaf Number (LN), Leaf Length (LL), Leaf Wide (LW), Fresh Leaf Weight (FLW), Dry Leaf Weight (DLW), Number of Daughter Corm (NDC), Fresh Weight of Daughter Corm (FWDC), and Dry Weight of Daughter Corm (DWDC). Dry Stigma Yield, Flower Number, Fresh Stigma Weight, Dry Stigma Weight, Stigma Length, Fresh Flower Weight and Dry Flower Weight traits were measured from 20 October to 20 November each year. Leaf Number, Leaf Length, Leaf Wide, Fresh Leaf Weight, and Dry Leaf Weight traits were evaluated every May 1st. Numbers of Daughter Corm, Fresh Weight of Daughter Corm and Dry Weight of Daughter Corm traits were measured every year from the beginning of July. To measure dry weight of plant materials, they were placed in an oven at a temperature of 50-55°C for one day and then the measured weight of the materials was considered as dry weight. After measuring the traits according to the above method, the mean of each plot in each year was used as the raw data.

DNA isolation

Total genomic DNA was extracted separately from 30 to 50 mg of saffron corm from each sample using BEIKI *et al.* (2011) method. The quality and quantity of extracted DNA were evaluated with a Nano-Drop® ND-1000 UV-Vis Spectrophotometer (Labtech International) and

in a 0.8% (w/v) agarose gel stained with ethidium bromide. DNA samples were diluted to 20 ng/µl and stored at −20°C for further analyses.

DNA amplification

In this assay, twenty-two different primer combinations were used for analyzing genetic diversity in studied saffron accessions. The genomic DNA were amplified in a final volume of 20 µl containing 5 µl DNA (20 ng), 2 mM dNTPs, 2 mM MgCl2, 0.2 unit of Taq polymerase, 2 mM of 10X PCR buffer and 1 pmol of each primer (forward and reverse). The cycling program began with an initial 5 min at 95°C followed by 45 cycles at 95°C for 45 s, 48–62°C for 45 s and 72°C for 2min plus a final 10 min at 72°C and storage at 4°C. Amplification products were separated by electrophoresis in 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. DNA molecular weight markers (1 kb, Promega) were then added to each gel. 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.

Scoring and data analysis

Average values for all traits in this study were calculated for further analyses. The data recorded on quantitative traits were averaged and analyzed for simple statistical approaches i.e. mean, minimum, maximum, standard deviation and coefficient of variation to determine the extent of genetic diversity among the studied genotypes.

DNA fingerprints were scored for the presence (1) or absence (0) of bands of various molecular weight sizes in the form of binary matrix. 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). The agronomic traits and molecular markers were considered as the dependent variables and independent variables, respectively. Stepwise regression was used in order to verify the linear relationship between independent and dependent variables, predict the value of the dependent variable based on the independent variable, remove the variables with negligible effect on the dependent variables and fit the best regression model. The probability level (P) for rejecting any association between a marker and an agronomical trait was 0.01 so it provided informative markers with a high significance level.

RESULTS

Descriptive statistics of the recorded data reflected a high level of variation for the quantitative traits in two years. All the traits were more or less, directly or indirectly, positively or negatively added to the yield, and they possessed key genetic status during the identification of productive genotypes. The basic statistical data (mean, minimum, maximum, standard deviation, coefficient of variation and variance) for every quantitative trait was calculated among all the genotypes in two years (Table 2). Pattern of variability among the genotypes was different for various agro-morphological traits. Maximum phenotypic variation was observed in Dry Weight of Daughter Corm (44.68%), Fresh Weight of Daughter Corm and (44.12%), Dry Stigma Yield (42.06%), and Number of Daughter Corm (41.46%) in year 2013, respectively. Also, maximum phenotypic variation was observed in Dry Stigma Yield (65.1%), Flower Number

(58.5%), Fresh Weight of Daughter Corm and (58.4%), Dry Weight of Daughter Corm (44.68%), and Number of Daughter Corm (55%) in year 2014, respectively.

In the initial assay, 25 SSR primers were employed to detect genotypic variation. Of those, 17 primers (68%) could yield well-defined and scorable polymorphic bands in all samples (Table 3). A total of 33 alleles were detected, ranging in size from 50 to 500 bp. Shannon's information index (I) and genetic diversity (h) ranged from 0.255 to 0.982 (average: 0.78) and 0.722 to 0.672 (average: 0.49), respectively (Table 3). The lowest PIC score was detected as 0.123 for primer combination SCC209 and the mean of PIC estimated 0.43 (Table 3).

Table 2- Descriptive statistics of saffron studied traits in two years.

statistics	Traits in 2013							
	DSY (kg/ha)	FN (m2)	FSW (mg/fl)	DSW (mg/fl)	SL (cm)	FFW (mg)	DFW (mg)	LN
Min	0.13	4.70	18.88	3.37	2.09	262.28	32.23	1.64
Max	1.51	21.19	34.10	7.20	4.93	389.92	49.90	8.20
Range	1.38	16.50	15.22	3.83	2.84	127.64	17.67	6.56
Mean	0.71	13.91	27.25	5.01	3.44	338.54	42.02	4.87
Std. error	0.02	0.24	0.19	0.04	0.03	1.53	0.22	0.09
Variance	0.09	15.99	10.87	0.55	0.33	674.78	13.73	2.15
Stand. dev	0.30	4.00	3.30	0.74	0.57	25.98	3.71	1.47
Coeff. var	42.06	28.74	12.10	14.86	16.60	7.67	8.82	30.11
	Traits in 2014							
Min	0.2	6.0	19.0	3.4	2.1	263.2	33.2	4.3
Max	5.7	89.4	36.6	7.6	5.8	399.8	50.8	10.5
Range	5.51	83.48	17.57	4.24	3.65	136.56	17.6	6.23
Mean	2.0	35.9	28.1	5.5	3.7	342.2	43.0	7.4
Std. error	0.1	1.2	0.2	0.0	0.0	1.6	0.2	0.1
Variance	1.7	440.3	13.2	0.7	0.5	772.5	14.2	1.5
Stand. dev	1.3	21.0	3.6	0.8	0.7	27.8	3.8	1.2
Coeff. var	65.1	58.5	12.9	15.3	19.0	8.1	8.7	16.7

Statistics	Traits in 2013						
	LL (cm)	LW (mm)	FLW (mg)	DLW (mg)	NDC	FWDC (gr)	DWDC (gr)
Min	7.43	1.29	100.05	27.08	0.60	4.27	1.41
Max	43.00	4.13	451.00	141.00	5.40	29.57	12.42
Range	35.57	2.84	350.95	113.92	4.80	25.30	11.01
Mean	19.76	2.41	246.46	72.55	2.54	13.29	5.27
Std. error	0.37	0.03	4.82	1.46	0.06	0.35	0.14
Variance	38.50	0.22	6683.57	616.33	1.10	34.40	5.55
Stand. dev	6.20	0.47	81.75	24.83	1.05	5.86	2.36
Coeff. var	31.40	19.32	33.17	34.22	41.46	44.12	44.68
	Traits in 2014						
Min	12.6	1.4	181.1	46.0	1.9	9.8	2.6
Max	43.9	3.9	411.1	120.0	21.3	108.1	38.5
Range	31.37	2.55	230	74.02	19.47	98.24	35.89
Mean	21.4	2.5	266.7	76.0	9.4	45.9	17.4
Std. error	0.3	0.0	2.6	0.9	0.3	1.6	0.6
Variance	22.1	0.2	1926.6	211.4	26.8	718.3	96.3
Stand. dev	4.7	0.4	43.9	14.5	5.2	26.8	9.8
Coeff. var	22.0	16.3	16.5	19.1	55.0	58.4	56.4

Table 3. SSR markers characteristics used in the study

Primer	Annealing temperature	No. of polymorphic bands	Shannon's Index (I)	Gene diversity (h)	Polymorphic information index (PIC)
SCA15	47.1	1	0.686	0.493	0.371
SCA109	64.8	3	0.808	0.357	0.335
SCA303	64.8	2	0.931	0.582	0.496
SCA319	61.5	1	0.685	0.492	0.371
SCA327	64.8	2	0.757	0.553	0.455
SCA381	57.5	1	0.351	0.199	0.179
SCA382	61.5	1	0.363	0.208	0.186
SCA393	64.8	3	0.865	0.393	0.359
SCA416	64.8	3	0.964	0.601	0.538
SCA504	64.8	2	0.884	0.583	0.506
SCA515	64.8	2	0.953	0.621	0.578
SCB109	64.8	2	0.944	0.644	0.577
SCB115	61.5	2	0.932	0.601	0.534
SCC13	64.8	2	0.898	0.455	0.435
SCC209	64.8	1	0.255	0.131	0.123
SCD17	64.8	3	0.961	0.676	0.627
SCD219	64.8	3	0.982	0.722	0.672
Total	---	34	13.22	8.31	7.34
Average	---	2	0.78	0.49	0.43

The results of stepwise regression analysis revealed a significant association between the traits and some of the studied loci in two years (Table 4). In stepwise regression, traits and markers were considered as the dependent variable and the independent variables, respectively. A total of 25 markers (alleles) that were significantly correlated and associated with studied traits, some of them were associated with several traits, and finally 8 markers were effective in phenotypic variation of traits. Other markers have no significant effect on the model; we can therefore say that the correlated markers can be used to identify superior genotypes in terms of the studied traits. The markers identified were varied from two markers for number of nodes to 11 markers for fresh weight and dry weight. These markers were negatively or positively correlated to traits. Other researchers are using regression analysis to identify the relationship between markers and studied traits and used them in the breeding program (BASAKI *ET AL.*, 2011; IPEK *ET AL.*, 2015; KHADIVI-KHUB, 2014; MARSAFARI *et al.*, 2014; RAKSHIT *et al.*, 2010).

Markers SCA382 and SCD2191 in year 2013, and SCA15 and SCA382 in year 2014 were associated with Dry Stigma Yield, they justify 93% and 92% of the phenotypic variation in two years, respectively (Table 4). Calculating standard β can be demonstrated the importance markers of each trait. SCA382 marker was the most important marker for DSY, FFW, LW, DLW, NDC and FWDC because this marker showed the most phenotypic variation of these traits. Also, SCD2193 marker was an important marker for the FLW and DLW and had an increasing effect (Table 4). According to the standardized β coefficients, some alleles had a lowering effect and some alleles had an enhancing effect of the studied traits (Table 4). The

markers with positive standardized β can be used to increase traits and the markers with negative standardized β can be used to reduce traits in breeding programs.

Table 4. Stepwise regression analysis of traits (dependent variable) and SSR markers (independent variables) in two years.

Traits[a]	Year	Number of informative markers	Informative markers*	R^2 adjusted (%)	Main marker with highest R2	B (regression coefficient)	R square changed
DSY	2013	2	SCA382-SCD2191	0.93	SCA382	-0.66**	0.75
(kg/ha)	2014	2	SCA15-SCA382	0.92	SCA15	-0.86**	0.86
FN	2013						
(m2)	2014	1	SCA15	0.76	SCA15	-0.89**	0.79
FSW	2013						
(mg/fl)	2014	1	SCD171	0.47	SCD171	-0.73*	0.53
FFW	2013	1	SCA382	0.42	SCA382	-0.70*	0.49
(mg)	2014	1	SCA382	0.57	SCA382	-0.79**	0.62
LN	2013						
	2014	1	SCA319	0.61	SCA319	0.809**	0.66
LL	2013	1	SCA382	0.65	SCA382	-0.83**	0.69
(cm)	2014						
LW	2013	1	SCA15	0.41	SCA15	-0.69*	0.47
(mm)	2014						
FLW	2013	2	SCD2193-SCA15	0.88	SCD2193	0.67**	0.77
(mg)	2014						
DLW	2013	2	SCD2193-SCA15	0.86	SCD2193	0.71**	0.80
(mg)	2014	2	SCA382-SCA319	0.69	SCA382	-0.95**	0.53
NDC	2013	1	SCA382	0.48	SCA382	-0.73*	0.54
	2014	1	SCD2193	0.87	SCD2193	0.94**	0.89
FWDC	2013	1	SCA382	0.48	SCA382	-0.74*	0.54
(gr)	2014	2	SCA382-SCD2193	0.92	SCA382	-0.60**	0.69
DWDC	2013	1	SCD2191	0.50	SCD2191	-0.74*	0.55
(gr)	2014	2	SCD2192-SCA382	0.92	SCD2192	-0.72**	0.80

[a]: :The abbreviation of traits is in the text.
* and **: Significant at 5% and 1% probability level, respectively.

DISCUSSION

Minimum, maximum, average, standard deviation and coefficient of variation to phenotypic traits are shown in Table 2. According to Table 2, for all evaluated traits, considerable variation among ecotypes was observed. Most of the phenotypic variation showed a high variation so we can say that there are plant genetic resources for use in breeding programs to improve these traits.

Five primer combinations produced the most bands: SCA109, SCA393, SCA416, SCD17 and SCD219 (3 bands) and the least bands were amplified by five primer combinations: SCA15, SCA319, SCA381, SCA382 and SCC209 (1 bands) while the average of polymorphic bands was

2.0. Similarly, CAIOLA *et al.* (2004) and BEIKI *et al.* (2010) observed 2.1 and 3.8 polymorphic bands per primer pair using RAPD primer, while MORAGA *et al.* (2010) obtained 4.8 polymorphic bands per primer in their ISSR study. These results indicated that SSR marker is a useful method to detect considerable polymorphisms in saffron accessions originated from different regions. On the other hand, we can report that saffron is not a monomorphic plant and accessions for breeding purposes can be identified. The previous studies showed contradictory results RUBIO-MORAGA *et al.* (2009), and ALAVI-KIA *et al.* (2008) could not detect any polymorphism among saffron accessions. The previous results led to classifying saffron as a monomorphic species, while SIK *et al.* (2008), BEIKI *et al.* (2010), NAMAYANDEH *et al.* (2013), BABAEI *et al.* (2014) and EROL *et al.* (2014; 2011) reported some diversities among saffron accessions.

Identification markers that be in coding the regions of these traits and entered in the regression model and were explanation of traits variations can be useful in breeding programs. Some of these markers were associated with more than one trait. According to a significant correlation between morphological traits, some of them had very close linkage or they were possibly controlled by pleiotropic effects. An important advantage of association analysis is that this method does not require preparation of segregating population, which takes more time, although it is better to use a multi-year phenotypic data. On the other hand, crossing over, which occurs during the preparation of segregating populations, limited the precise positioning. The efficiency of these methods has been shown in identifying and mapping the controlling gene of Mendelian traits (BRESEGHELLO and SORRELLS, 2006). It also uses informative markers associated with traits; chromosomal location can be a particularly effective step taken in the initial selection of genotypes with high yield. Also, informative markers that are identified in association analyses and showed a high phenotypic variation with high R^2 in regression model can be isolated and cloned and used in breeding program. Alignment of databases with existing sequence was identified, as well as candidate genes, which are very similar to markers. It can also be used over the desired sequence to designed primers (SCAR) for interesting traits and in marker-assisted selection in breeding programs.

There was a high association of the expression of the informative markers between two years. As QUARRIE *et al.* (2005) stated that may have resulted from preferential expression of QTL under drought stress conditions. However, markers SCA382 and SCA15 were found to be associated with some traits in both years. Therefore, these markers were considered to be relatively more reliable (Table 4). Some of these informative markers can be separated by gel electrophoresis and hopefully considered as candidate markers for scanning the genome for related agronomic traits, mapping, and breeding programs. Also, SCAR markers can be obtained from sequenced informative marker fragments in order to be used in marker-assisted selection (MAS) (RUAN, 2010).

CONCLUSION

Informative fragments could be successfully cloned and sequenced for polymorphic diagnostics. Hopefully some of these markers will be used for MAS in future saffron breeding programs. Where crossing between more genetically distant individuals will increase the chance of transgressive segregation in their progeny. These markers could therefore be used to choose parents for the development of the mapping populations. SSR primers SCA382 and SCA15 showed fragments with the highest association with the traits. These primers have been found useful for the study of genetic diversity and association analyses in saffron. Stable marker,

consistently identified across two years, can be separated by gel electrophoresis and considered as candidate markers for scanning the genome for related agronomical traits.

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IDENTIFIKACIJA MARKERA POVEZANIH ZA SVOJSTVIMA ZA MARKER-ASISTIRANU SELEKCIJU ŠAFRANA

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Izvod

Određivanje veze između molekularnih markera i agronomskih osobina predstavlja odličan alat za selekciju pomoću markera. U ovoj studiji, multivarijantna stepenasta regresiona analiza korišćena je za procenu veze između SSR markera i nekih agronomskih osobina u ekotipovima šafrana. Za analize asocijacija korišćene su dvogodišnje prosečne vrednosti za izmerene osobine. Rezultati postupne analize regresije otkrili su značajnu asocijaciju između osobina i proučavanih lokusa. Identifikovano je ukupno 25 SSR markera. Markeri SCA382, SCA15 i SCD219 bili su povezani sa većinom osobina, i mogu se koristiti kao kandidat markeri u selekciji pomoću molekularnih markera.

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