**Saffron (Crocus sativus L.), a monomorphic or polymorphic species?**

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**Abstract**

Saffron (Crocus sativus L.) which contains exceptional anti-cancer properties is presently the world’s most expensive spice. Iran is known as the original habitat of Crocus L. and a significant source of high-quality cultivated saffron production and export. Considering the importance of this species, we used 27 microsatellite markers to assess molecular variability and discriminating capacity of markers regarding their effectiveness in establishing genetic relationships in Iranian Crocus ecotypes. Thirty eight Iranian cultivated saffron ecotypes and 29 wild allies were evaluated in this research. The results from molecular analyses, including a molecular phylogenetic network and RB analysis, revealed two major groups and five subgroups, regardless of their geographical origins. Also, the results showed a clear distinction between C. sativus and other species of Crocus genus, taking into account their close relationship with C. speciosus and C. hausknechtii, which are assumed to be the two closest relatives of Iranian cultivated saffron among species studied. In this paper, we observed for the first time extensive genetic diversity among Iranian C. sativus despite their asexual reproduction. Considering suitable climatic conditions in Iran for cultivating saffron and the country’s leading high-quality production of Crocus sativus worldwide, studies on great genetic variability among Iranian C. sativus ecotypes as well as wild relatives native to Iran will further highlight the value of this crop. In addition, our results provide valuable information for genetic improvement, reduction of strong genetic erosion, and conservation of costly heritable resources of C. sativus in future breeding programs.

**Additional key words**: medicinal plants; SSR markers; genetic variability.

**Introduction**

The most prominent member within the Crocus series and the whole genus is C. sativus L. The precious aromatic and medicinal species C. sativus L. (saffron) is of prime economic importance (Rios et al., 1996; Ferrence & Bendersky, 2004). In recent years, researchers have discovered and well documented exceptional therapeutic properties such as anticancer, antimutagenic and antioxidant qualities in C. sativus and some other Crocus species (Abdullaev & Espinosa-Aguirre, 2004; Chryssanthi et al., 2007), showing that the species can have potential pharmaceutical applications. All species of Crocus are diploid but Crocus sativus is a triploid (2n = 3x = 24) which is propagated vegetatively by means of bulbs or corms (Brighton, 1977; Mathew, 1977). The cultivation of saffron dates back to 1500-2500 BC in Iran, Greece, India, China, the Me-

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Abbreviations used: AFLP (amplified fragment length polymorphism); He (expected heterozygosity); Ho (observed heterozygosity); IRAP (inter retrotransposon amplified polymorphism); ISSR (inter simple sequence repeat); PIC (polymorphism information content); RAPD (random amplified polymorphic DNA); RB (repeated bisection); SRAP (sequence related amplified polymorphism); SSR (simple sequence repeat).
diterranean basin, and Eastern Europe (Negbi et al., 1989; Beiki et al., 2010). Iran is the largest producer accounting for almost 80% of the total world production (Ahmad et al., 2011). Ranked first in the world, Khorasan province, Iran, is specifically the most ideal place for the growth and production of cultivated saffron (Ahmad et al., 2011). Iran is also the native habitat of eight *Crocus* species besides *C. sativus*, four of which are exclusively indigenous to this country.

Morphological comparisons of cultivated saffron ecotypes have revealed some differences in intensity of flower color, viability, pollen size, and number of style branches and stamens (Grilli-Caiola et al., 2001). Such comparisons suggest the existence of genetic variability in *C. sativus* ecotypes or commercial varieties. Furthermore, the actual genetic diversity existing in *C. sativus* is still unknown. Over the past centuries, the problem of intense genetic erosion in *Crocus* due to loss of land surface allocated to this crop in many areas of the world has further reduced the genetic variation of this crop (Abdullaev & Espinosa-Aguirre, 2004).

Because native ecotypes of cultivated saffron and their wild relatives are enormous and valuable genetic resources of each peculiar habitat, there is an urgent need to identify and evaluate their genetic variation to prevent genetic erosion. Molecular markers may provide a reliable tool for measuring genetic divergence of plant cultivars as they remain unaffected by environmental factors and developmental stages of plants. Considering the potentials of DNA-based markers, Simple Sequence Repeats (SSR) can considerably help to assess diversity and intraspecific relationships across other markers. Studying SSR markers, Rubio-Moraga et al. (2009) evaluated 43 saffron isolates by 15 microsatellite primers, none of which was polymorphic in the population under study. On the contrary, Nemati et al. (2012) detected a good level of polymorphism by 12 microsatellite markers within 50 Iranian individuals of *Crocus sativus*. The purpose of this study was to investigate the spectrum of genetic diversity within Iranian *Crocus* spp., to reveal the phylogenetic relationships of 67 *Crocus* collected from different sites of Iran using 27 microsatellite markers, to propose a strategy for broadening the genetic base for future breeding of this valuable crop.

**Material and methods**

**Plant material**

Thirty eight *C. sativus* from five geographically separated populations of Iran (Ghaen, Gonabad, Estahban and Ferdos) and 29 allies including species of *C. haussknechtii*, *C. caspius*, *C. speciosus* and *C. cancellatus* collected from Kaleybar, Alasht, Veresk, Islam Abad, Reno and Neka were used in this study (Table 1 and Fig. 1).

**Molecular analysis**

Fresh leaves were frozen using liquid nitrogen and kept at −80°C. High molecular weight genomic DNA was extracted from fresh leaves of *Crocus* using DNeasy Plant Mini kit (Qiagen, Germany). The quality of the extracted DNA was verified on a 1% agarose gel and the amount of total genomic DNA obtained

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Species</th>
<th>Ploidy level</th>
<th>City/Area</th>
<th>Province</th>
</tr>
</thead>
<tbody>
<tr>
<td>Go1 to Go8</td>
<td><em>C. sativus</em></td>
<td>3X</td>
<td>Gonabad</td>
<td>Khorasan</td>
</tr>
<tr>
<td>Gh1 to Gh5</td>
<td><em>C. sativus</em></td>
<td>3X</td>
<td>Ghaen</td>
<td>Khorasan</td>
</tr>
<tr>
<td>Es1 to Es14</td>
<td><em>C. sativus</em></td>
<td>3X</td>
<td>Estahban</td>
<td>Fars</td>
</tr>
<tr>
<td>Fe1 to Fe11</td>
<td><em>C. sativus</em></td>
<td>3X</td>
<td>Ferdos</td>
<td>Khorasan</td>
</tr>
<tr>
<td>Ve1 to Ve5</td>
<td><em>C. speciosus</em></td>
<td>3X</td>
<td>Veresk</td>
<td>Mazandaran</td>
</tr>
<tr>
<td>Al1 to Al4</td>
<td><em>C. speciosus</em></td>
<td>2X</td>
<td>Alasht</td>
<td>Mazandaran</td>
</tr>
<tr>
<td>Ka1 to Ka4</td>
<td><em>C. haussknechtii</em></td>
<td>2X</td>
<td>Kaleybar</td>
<td>Azarbayjan</td>
</tr>
<tr>
<td>Is1 to Is4</td>
<td><em>C. haussknechtii</em></td>
<td>2X</td>
<td>Islam abad</td>
<td>Kermanshah</td>
</tr>
<tr>
<td>Re1 to Re3</td>
<td><em>C. haussknechtii</em></td>
<td>2X</td>
<td>Reno</td>
<td>Iram</td>
</tr>
<tr>
<td>Ne1 to Ne5</td>
<td><em>C. caspius</em></td>
<td>2X</td>
<td>Neka</td>
<td>Mazandaran</td>
</tr>
<tr>
<td>Re1 to Re4</td>
<td><em>C. cancellatus</em></td>
<td>2X</td>
<td>Reno</td>
<td>Mazandaran</td>
</tr>
</tbody>
</table>
was quantified using a NanoDrop (ND-1000) Spectrophotometer (Nanodrop Technologies, http://nanodrop.com). PCR amplifications were performed using the 27 \textit{C. sativus} microsatellite (SSR) markers developed by Rubio-Morga \textit{et al.} (2009) and Nemati \textit{et al.} (2012). Each 25 µL PCR reaction contained 2.5 µL of 1x PCR buffer, 2 µL of 25 mM MgCl$_2$, 1.5 µL of 10 mM dNTPs, 1 µL of each primer (10 pmol µL$^{-1}$), 0.2 µL of Taq DNA polymerase (5 U µL$^{-1}$) (Fermentas), 14.8 µL of double distilled water, and 2 µL of extracted DNA (ca. 20 ng). The cycling conditions were: initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing temperature at 47-55°C and extension temperature 72°C for 2 min, and a final extension at 72°C for 7 min. PCR products were visualized by 4% of metaPhor Agarose gel (Lonza, Rockland, USA). A 50 bp DNA ladder (Fermentas, Sankt Leon-Rot Germany) was used to measure the size of the alleles. The primers names, sequences, repeat motifs, and annealing temperatures are shown in Table 2.

Considering the electrophoresis results, DNA fragments were scored on a binary scale: present (1) or absence (0). Due to the triploidy of \textit{C. sativus}, different parameters of genetic diversity were measured using the R package POLYSAT version 1.10 (Clark & Jasieniuk, 2011).

Two methods were performed to estimate the genetic distances between samples: phylogenetic network and partial repeated bisection (RB) analysis which were conducted using SplitsTree vers. 4.11.3 (Huson & Bryant, 2006) and gCluto v. 1.0 program (Rasmussen & Karypis, 2004) respectively.

A Bayesian clustering approach was utilized to assess the genetic structure of the ecotypes using STRUCTURE vers. 2.3.3 Software (Pritchard \textit{et al.}, 2000). In this model, the population of the genetic structure was characterized by admixture model and correlated allele frequencies. Twenty independent runs were performed for each value of \(K\) ranging from 2 to 10, and the DK method (Evanno \textit{et al.}, 2005) was used to choose the most likely value of \(K\).
Table 2. Information of twenty-four polymorphic SSR loci

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ → 3’)</th>
<th>Repeat motif</th>
<th>Tm (°C)</th>
<th>N&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Ho&lt;sup&gt;4&lt;/sup&gt;</th>
<th>He&lt;sup&gt;5&lt;/sup&gt;</th>
<th>PIC&lt;sup&gt;6&lt;/sup&gt;</th>
</tr>
</thead>
</table>
| CSMIC14 | F: CCTTGCTTTGAAAATGCTGG  
R: TTGCAGAATTTCCCTGCCGTT | (T)<sub>11</sub> (TCTTCC)<sub>2</sub> (T)<sub>13</sub> | 60 | 3 | 0.95 | 0.54 | 0.52 |
| CSMIC23 | F: GTCACCTAATCGGTGGT  
R: AATCTATCTAAGGCTCTCA | (AAG)<sub>3</sub> (TCATA)<sub>2</sub> | 50 | 1 | 0.14 | 0.14 | 0.11 |
| CSMIC25 | F: GTCTCCTCGTATCTCCTTGA  
R: ACCTCAGAGAATGCAGCAAT | (TCT)<sub>3</sub> | 50 | 1 | 0.08 | 0.15 | 0.1 |
| CSMIC36 | F: GCTAGCAGAATCAGATGACCA  
R: AGTGCATTCTACCTACGCCTCA | (CAC)<sub>5</sub> | 2 | 0.87 | 0.24 | 0.43 |
| CSMIC44 | F: CAGTGCTCGGCTGAATGTGA  
R: ACTGCTGACGCTGCAACTT | (GCTG)<sub>2</sub> (CCT)<sub>12</sub> (TTTC)<sub>2</sub> | 60 | 2 | 0.43 | 0.42 | 0.15 |
| CSMIC46 | F: GTCAAGTGCTGAGGAGGA  
R: TGGATACGCTGCATATCTCA | (AG)<sub>18</sub> | 58 | 1 | 0.09 | 0.20 | 0.08 |
| CSMIC47 | F: ACCAGTGCATGGATGCACAT | (CTAT)<sub>9</sub> | 60 | 2 | 0.02 | 0.10 | 0.49 |
| CSMIC50 | F: TAAACTCTGCGGAGGCAGTGG  
R: GGAAGACAAAGTGCCGGGTTGA | (AG)<sub>21</sub> | 60 | 2 | 0.18 | 0.15 | 0.29 |
| CSMIC53 | F: GCAGATCAGCAGCTGACCGGTT  
R: CAGTGCTCAGCTGCAACTT | (AGC)<sub>3</sub> | 62 | 2 | 0.2 | 0.19 | 0.17 |
| CSMIC55 | F: AGCAAAGGGCCACACATTCA  
R: AGCTGTCAGTCCAATCATCAAC | (CTAT)<sub>9</sub> | 60 | 2 | 0.02 | 0.10 | 0.49 |
| CSMIC59 | F: GAAATATGTTGAGAGGCCGGA  
R: AAGAGAGATTTAATAAGTCCGA | (AG)<sub>9</sub> (AG)<sub>12</sub> | 55 | 2 | 0.35 | 0.18 | 0.39 |
| CSMIC62 | F: CCAACTTGGAGGAGCGGCT  
R: AGAAGCCTGAGTAAGTGA | (GA)<sub>3</sub> (AG)<sub>7</sub> | 60 | 3 | 0.65 | 0.56 | 0.55 |
| ABRII/Cs 2 | F: ATACGGTAACTCAGGGAAG  
R: AGTAATCCAGCGTCAAGGT | (GAA)<sub>7</sub> | 53 | 3 | 0.65 | 0.56 | 0.53 |
| ABRII/Cs 8 | F: GTGAATGAATGGGATATATGGC  
R: CTTCCAACCTGGAATATAATC | (AG)<sub>12</sub> | 53 | 3 | 0.92 | 0.57 | 0.54 |
| ABRII/Cs 10 | F: GGATGTACTTAGGTGTG  
R: GGAAACCCTACTAGGT | (AG)<sub>26</sub> | 50 | 2 | 0.85 | 0.53 | 0.51 |
| ABRII/Cs 11 | F: CCCAACGTGGACTCCTCACAATCT  
R: GTGTGTTGATGTTGCTGCG | (CT)<sub>13</sub> | 55 | 3 | 0.89 | 0.58 | 0.55 |
| ABRII/Cs 20 | F: CAACTTTACATAGTGAGGC  
R: GTATTTGTTGCTGAGCTTAC | (AG)<sub>7</sub> (GAA)<sub>10</sub> | 55 | 2 | 0.90 | 0.55 | 0.50 |
| ABRII/Cs 21 | F: TACCCTATAAAAGAGTGGACA  
R: GCTGCGCTAGTAAATGCTGAAG | (AATTAG)<sub>2</sub> | 52 | 2 | 0.52 | 0.45 | 0.40 |
| ABRII/Cs 28 | F: AACCACTAGGGAAAGGAC  
R: GGTGAGAATACCTTACCGGT | (ACCGCG)<sub>2</sub> | 52 | 2 | 0.79 | 0.54 | 0.51 |
| ABRII/Cs 30 | F: TCTCTCTAGATTACAATCCCTC  
R: CTGTTGTTGAAAGGATACTA | (ACTAAT)<sub>2</sub> | 50 | 2 | 0.15 | 0.12 | 0.3 |
| ABRII/Cs 39 | F: CTTTAGCTTTATGATGTTGTC  
R: TCCCGGTATGTAACCTATGTA | (GA)<sub>16</sub> | 50 | 2 | 0.09 | 0.13 | 0.11 |
| ABRII/Cs 42 | F: ATTAACACCCGGTCACCTAGA  
R: GAAGGTATCTTCTCCCTCGT | (GAA)<sub>14</sub> | 50 | 2 | 0.07 | 0.10 | 0.09 |
| ABRII/Cs 48 | F: TCCCTAAACTTGACTTGAGA  
R: TCCCGGTATGTAACCTATGTA | (CT)<sub>15</sub> | 50 | 2 | 0.16 | 0.21 | 0.18 |
| ABRII/Cs 56 | F: AGAAGAGAGAGACAGAGAAAAC  
R: GTAGATCATCCACACTATCC | (TTAGGG)<sub>2</sub> | 50 | 2 | 0.08 | 0.11 | 0.2 |

<sup>1</sup> Primers were developed by Rubio-Morga et al. (2009) and Nemati et al. (2012).  
<sup>2</sup> Tm: PCR annealing temperature.  
<sup>3</sup> N: number of alleles.  
<sup>4</sup> Ho: observed heterozygosity.  
<sup>5</sup> He: expected heterozygosity.  
<sup>6</sup> PIC: polymorphism information content.
Analysis of molecular variance (AMOVA) was conducted using ARLEQUIN version 3.5 (Excoffier & Lischer, 2010). AMOVA was calculated over all populations to estimate the intrapopulation and interpopulation variation (Weir & Cockerham, 1984; Excoffier et al., 1992). The significance level of $F_{ST}$ statistics was determined through a nonparametric permutation procedure with 1,000 randomizations implemented in ARLEQUIN. Nei’s standard genetic distance was calculated using the same program. Statistical calculations and graphics for $F_{ST}$ (Weir & Cockerham, 1984) were calculated using R v. 2.12.1 (cran.r-project.org).

Results

Genetic diversity

Table 2 shows that among all 27 primer pairs, 24 were recognized as polymorphic markers producing a total of 50 alleles with a mean of 2.08 per locus. Average observed heterozygosity ($H_o$) was 0.42 ranging from 0.08 to 0.95, and expected heterozygosity ($H_e$) ranged from 0.10 to 0.58 with an average of 0.31 per locus. The polymorphism information content (PIC) value, which is commonly used to estimate the informativeness of a marker or the discriminatory power of the locus, ranged between 0.1 in CSMIC25 and 0.54 in ABRII/Cs 8 with an average of 0.35 per marker. ABRII/Cs 11, ABRII/Cs 8 and ABRII/Cs 2 markers had the highest PIC values among others, representing a potential to be markers of choice in future breeding programs. Positive relationships between genetic diversity, PIC values, and number of alleles were also assessed (Table 2).

Phylogenetic relationships

The Neighbor-Net analysis results divided the 67 Iranian Crocus ecotypes into five main clusters (Fig. 2). Most of the cultivated and wild species of Crocus showed a considerable distribution on the top and bottom of the derived dendrogram, respectively, with a slight tendency to their geographical origin. The phylogenetic tree shows the out-groups, indicating the intense genetic admixture of these ecotypes. The dendrogram generated from RB cluster analysis showed the existence of five main clusters. The maximum and minimum amounts of samples belonged to clusters 5, 1 and 2, respectively (Fig. 3). All cultivated saffron samples fell under only one cluster (5) with a close genetic distance to clusters 1 and 2.

Structure analysis

The grouping of individuals based on Bayesian clustering analysis confirmed the grouping we observed in the neighbor net analysis (Fig. 2) and RB method (Fig. 3). The most likely value of delta K was 5, presenting a division of genetic variation into five clusters as well (Figs. 4a,b). A pairwise comparison between 11 geographical populations from Iran based on the microsatellite allele frequencies showed that all of the domesticated saffron populations differed significantly from their wild relatives (Figs. 5a,b). Moreover, a high level of intrapopulation differentiation was found in C. speciosus (Ve) and C. sativus (Go), whereas a low level of intrapopulation differentiation was discovered in C. hausknechtii (Re) (Fig. 5a). The results of AMOVA analyses performed with SSR markers are presented in Table 3. A high degree of variation was due to differences among individuals within the populations (52.38%), which represent a much higher value than the variations existing across populations (6.55%).

Discussion

C. sativus as cultivated crocus is a sterile species with distinct morphological and growing characteris-
Figure 2. Phylogenetic relationships inferred based on SSR data using the distance-based Neighbor-Net method.
tics. Despite the existence of different commercial Crocus ecotypes and performance of different studies on evaluation of genetic analysis in Crocus species based on various molecular markers such as IRAP (Alavikia et al., 2008); RAPD (Beiki et al., 2010); RAPD and SRAP (Keify & Beiki, 2012); RAPD and ISSR (Rubio-Moraga et al., 2009); ISSR (Rubio-Moraga et al., 2010); AFLP (Siracusa et al., 2012; Erol et al., 2014) and SSR (Rubio-Moraga et al., 2009; Nemati et al., 2012), the actual genetic diversity present in C. sativus is still an open question. In this study, 27 microsatellite markers were tested to measure level of polymorphism and investigated the genetic relationship and structure among Iranian Crocus ecotypes.

The 24 out of 27 microsatellite primer pairs generated clear polymorphism bands among 67 accessions from four species of the Crocus genus. The mean number of alleles was 2.08 per locus with a total of 50 alleles. The average PIC value was 0.38 ranging from 0.1 to 0.54 (Table 2). Nemati et al. (2012) observed that a total of 27 alleles with a mean of 2.60 per locus and the mean of PIC across all loci varied from 0.1 to 0.54 with an average of 0.34 in their SSR analysis, which is apparently compatible with our present results. Moreover, our results showed a new set of 12 polymorphic microsatellite loci out of 15 monomorphic micro-
Figure 5. Average number of pairwise differences (a) and matrix of FST (b) shows the comparison among populations from ten geographical localities (see Fig. 1) based on microsatellite allele frequencies.
satellite markers reported by Rubio-Moraga et al. (2009), emphasizing that the molecular markers used in the present study can serve as useful tools for detecting the level of polymorphism in *Crocus* populations studied. It would be interesting to extend the study of genetic diversity using a larger number of SSR loci and sample size (Eckert et al., 2008). The transmissibility of SSR loci isolated from *C. sativus* into wild relatives can implicate the conservation of genic regions through the *Crocus* genus. This result can significantly help to reduce costs of genetic analysis in future breeding researches (Moretzsohn et al., 2004).

The dendrograms generated from Neighbor-Net and RB cluster analyses revealed five major groups (Figs. 2 & 3), with slight correlation between genetic distance and geographical origin. The cluster analysis results obtained in the present study are broadly consistent with the findings of earlier diversity studies based on RAPD and ISSR (Rubio-Moraga et al., 2009) and AFLP data (Erol et al., 2014), showing a separation of species independent of geographical origin. Our dendrogram indicated that *Crocus* species appeared to be closer to *C. hausknechtii* and *C. speciosus*.

The model-based structure analysis revealed the presence of five populations among the collected samples (Fig 4). The grouping patterns obtained from the Bayesian clustering approach was confirmed using the two distance-based phylogeny analyses (Figs. 2, 3 & 4). Moreover, no tendency between grouping and origin of *Crocus* ecotypes obtained from the model-based method was in agreement with the Neighbor Net analysis, demonstrating that the *Crocus* genotypes have a complex genetic structure. Erol et al. (2014) also reported two populations of saffron collected from different locations across Turkey and two East Aegean islands independent of their origins based on the model-based structure analysis.

Analysis of molecular variance (AMOVA) indicated that intrapopulation differentiation (52.38%) was higher than interpopulation differentiation (6.55%) (Table 3). AMOVA analysis in another study showed a high heterogeneity of intrapopulation (55.5%) as opposed to a slightly lower heterogeneity of interpopulation (44.5%) (Alavi-Kia et al., 2008). Furthermore, the highest level of intrapopulation variation referred to *C. speciosus* (Ve) and *C. sativus* (Go) is displayed in Fig. 5a. It is likely that cultivated *Crocus* isolates in Iran, particularly *C. sativus* (Go), display high genetic variation within population as well as *C. speciosus* (Ve). However, the lowest level of intrapopulation variation was observed in *C. hausknechtii* (Re), which showed no polymorphism among other wild relatives of *C. sativus*, supporting the case of no amplification of conserved zones of microsatellites studied in this ecotype.

The microsatellite markers used in the present study are potentially useful for evaluation of genetic diversity and population structure in *C. sativus* as well as detecting genetic relationships among Iranian *Crocus* species. Like other studies, we observed a reasonable polymorphism among Iranian *C. sativus* germplasms (Beiki et al., 2010; Keifi & Beiki, 2012) which may be due to suitable climatic conditions for growth and development of this valuable crop in Iran. The use of a large number of polymorphic markers is vital for accurate assessment of genetic variation among different *Crocus* species according to their geographical origin and ploidy level, formation of core collection, and construction genetic map. These markers may allow us to trace back the evolutionary history of saffron, especially in the case of detection and identification of the parental species and geographical origin of *C. sativus*.

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References


