Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
http://dx.doi.org/10.5424/sjar/2014123-5564

Spanish Journal of Agricultural Research 2014 12(3): 753-762
ISSN: 1695-971X
eISSN: 2171-9292

RESEARCH ARTICLE

OPEN ACCESS

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

Zahra Nemati[1]*, Mohsen Mardi[2], Parastoo Majidian[3], Mehrshad Zeinalabedini[2], Seyed M. Pirseyedi[4] and Moheb Bahadori[5]

[1] Young Researcher Club. IslamicAzad Univ., Shiraz branch. Sadra Road. 4-71993 Shiraz, Iran. [2] Agriculture Biotechnology Research Institute of Iran (ABRII). Seed and Plant Improvement Institutes Campus. Mahdasht Road. 31535-1897 Karaj, Iran. [3] Young Researcher Club. Islamic Azad Univ. Sari branch. Farahabad Road. 48186-19318 Sari, Iran. [4] Department of Plant Sciences. North Dakota State Univ. P.O. Box 6050. Fargo. ND 58180-6050, USA. [5] Department of Plant Protection. College of Agriculture. Shahid Chamran Univ. Golestan Road. 61351-831351 Ahzav, Iran

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-

* Corresponding author: za.nemati@gmail.com
Received: 06-01-14. Accepted: 23-07-14.

Abbreviations used: AFLP (amplified fragment length polymorphism); He (expected heterozigosity); Ho (observed heterozigosity); 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 1. Species name, ploidy level, and sampling location of *Crocus sativus* and its allies studied

Sample name	Speciees	Ploidy level	City/Area	Province
Go1 to Go8	*C. sativus*	3X	Gonabad	Khorasan
Gh1 to Gh5	*C. sativus*	3X	Ghaen	Khorasan
Es1 to Es14	*C. sativus*	3X	Estahban	Fars
Fe1 to Fe11	*C. sativus*	3X	Ferdos	Khorasan
Ve1 to Ve5	*C. speciosus*	3X	Veresk	Mazandaran
Al1 to Al4	*C. speciosus*	2X	Alasht	Mazandaran
Ka1 to Ka4	*C. speciosus*	2X	Kaleybar	Azarbayjan
Is1 to Is4	*C. hausknechtii*	2X	Islam abad	Kermanshah
Re1 to Re3	*C. hausknechtii*	2X	Reno	Iram
Ne1 to Ne5	*C. casoius*	2X	Neka	Mazandaran
Re1 to Re4	*C. cancellatus*	2X	Reno	Mazandaran

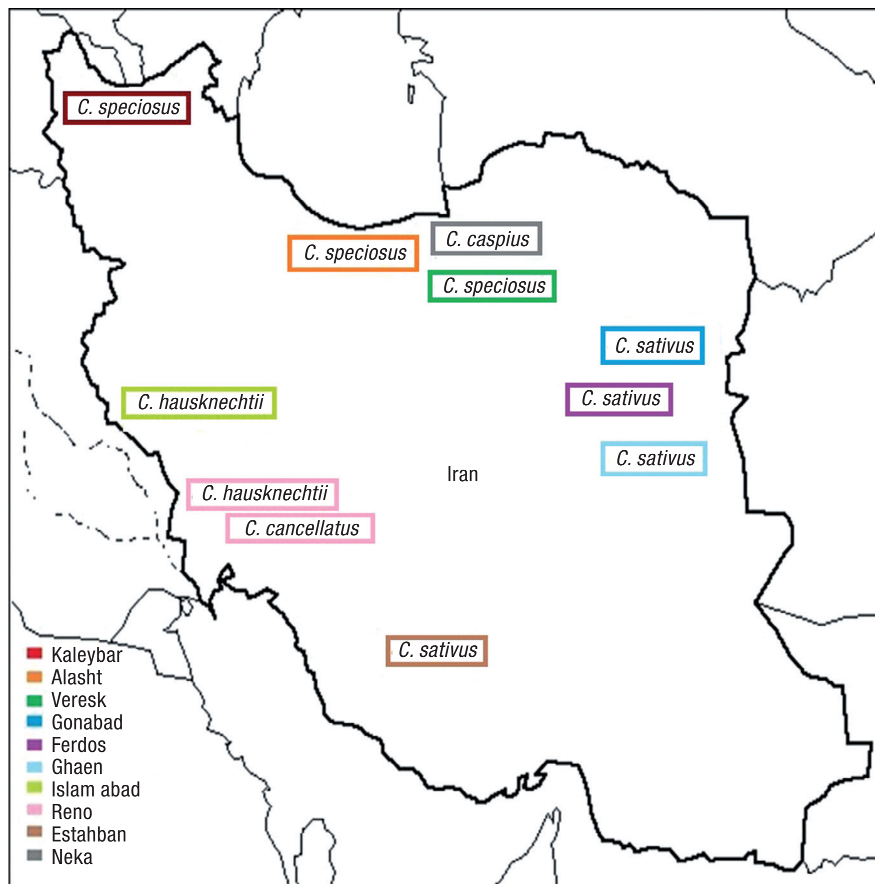

Figure 1. Map of 67 collected saffron (Crocus sp.) ecotypes from ten different localities in Iran.

was quantified using a NanoDrop (ND-1000) Spectrophotometer (Nanodrop Technologies, http://nanodrop.com). PCR amplifications were performed using the 27 *C. sativus* microsatellite (SSR) markers developed by Rubio-Morga *et al*. (2009) and Nemati *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 *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 *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 *et al*., 2005) was used to choose the most likely value of *K*.

Table 2. Information of twenty-four polymorphic SSR loci

Primers[1]	Sequence (5' → 3')	Repeat motif	Tm (°C)[2]	N[3]	*Ho*[4]	*He*[5]	PIC[6]
CSMIC14	F: CCTTGTCTTGAACGAATGTCTG R: TTGCAGAATCCTTGGCCTTA	$(T)_{11}$ $(TCTTCC)_2$ $(T)_{13}$	60	3	0.95	0.54	0.52
CSMIC23	F: GTCACTTACATGTTGGTGT R: AATTCTATTCCAAGGCTCCA	$(AAG)_5$ $(TCATA)_2$	50	1	0.14	0.14	0.11
CSMIC25	F: GTCTCCTTCGCTATCTCCTTGA R: ACCTTCAAGAAGATCAGCAAT	$(TCT)_5$	50	1	0.08	0.15	0.1
CSMIC36	F: GCTAGCAGAATCACATGATCCA R: AGTGCATTCATCTCACCTCTCA	$(CAC)_5$	50	2	0.87	0.24	0.43
CSMIC44	F: CAGTGCTTCGGCTGAATGTGAA R: ACTGCTGGACGGTGCAACTT	$(GCTG)_4$ $(CCT)_{12}$ $(TTTTC)_2$	60	2	0.43	0.42	0.15
CSMIC46	F: GTACAGTGCTGAAGAGGAGGA R: TGGATACGCTGCACGTATCTCA	$(AG)_{18}$	58	1	0.09	0.20	0.08
CSMIC47	F: ACCAGGTCAGTTGATGCCTCAT R: CAGTGTAGCTACTTAGACAGT	$(CTT)_9$ $(TTCCT)_6$ $(CTT)_9$	48	2	0.11	0.23	0.47
CSMIC50	F: TAACCTCGTCGGAGCGGTGGA R: GGAGCAACAATGGCGGTGGAA	$(AG)_{21}$	60	2	0.18	0.15	0.29
CSMIC53	F: GCAGAATCACTGCTGGACGGGT R: CAGTGCTTCGGCTGAATGTGAA	$(AGC)_9$	62	2	0.2	0.19	0.17
CSMIC55	F: AGCAACAGAGGCACACATTCA R: AGCTGTCAGTCCAATCATCAAC	$(CTAT)_9$	60	2	0.02	0.10	0.49
CSMIC59	F: GAATATTGTTGATGAGGCCGGA R: AAGAGAGATATTAAATAAGTCGCA	$(AG)_8$ $(AG)_{12}$	55	2	0.35	0.18	0.39
CSMIC62	F: CCAATCTGAGGACGGGCT R: AGAAGCGTGATGAAGTGA	$(GA)_8$ $(AG)_7$	55	3	0.65	0.56	0.55
ABRII/Cs 2	F: ATACGGTAACATCAGGAAG R: AGTAATCCACGCGTCAAGGT	$(GAA)_7$	55	3	0.65	0.56	0.53
ABRII/Cs 8	F: GTGTAATGAATGGGATATATGGC R: CCTTCCAACTGTGAAATAATTCC	$(AG)_{12}$	55	3	0.92	0.57	0.54
ABRII/Cs 10	F: GGATGTACTTAGGTTGTG R: GGAAACCCTAACTAGGT	$(AG)_{26}$	50	2	0.85	0.53	0.51
ABRII/Cs 11	F:CCCAACTGACCTTCCAACTG R: GTTGTTATGATGGTCTGGCC	$(CT)_{15}$	55	3	0.89	0.58	0.55
ABRII/Cs 20	F: CAATCTTTACATAGTGAGGC R: GTATTCTGGTCAGTTCAGTG	$(AAG)_7$-$(GAA)_{10}$	55	2	0.90	0.55	0.50
ABRII/Cs 21	F: TACCCTATAAAGAGTGGACA R: GCTGCCTAGTAATGTGTAAG	$(AATTAG)_2$	55	2	0.52	0.45	0.40
ABRII/Cs 28	F: AACCACTGAGGAAGGAC R: GGTAGAATACCTTATCGGTT	$(ACCGCG)_2$	55	2	0.79	0.54	0.51
ABRII/Cs 30	F: TCTCTCATGTTACAATCCTC R: CTGTGTTGAAGGGATATCTA	$(ACTAAT)_2$	50	2	0.15	0.12	0.3
ABRII/Cs 39	F: CTTTAGCTGTTATGATGGTC R: TCCCGGTATGTAACTATGTA	$(GA)_{16}$	50	2	0.09	0.13	0.11
ABRII/Cs 42	F: ATTAACACCGGTCACTAGA R: GAAGGTATCTCTCTTCGTTT	$(GAA)_{14}$	50	2	0.07	0.10	0.09
ABRII/Cs 48	F: TCCCTAAACTTGTACTGAGA R: TCCCGGTATGTAACTATGTA	$(CT)_{15}$	50	2	0.16	0.21	0.18
ABRII/Cs 56	F: AGAAGAGAGAGACGAGAAAC R: GTACATGAATCCAACTATCC	$(TTAGGG)_2$	50	2	0.08	0.11	0.2

[1] Primers were developed by Rubio-Morga *et al.* (2009) and Nemati *et al.* (2012). [2] Tm: PCR annealing temperature. [3] N: number of alleles. [4] *Ho:* observed heterozigosity. [5] *He:* expected heterozygosity. [6]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 (*Ho*) was 0.42 ranging from 0.08 to 0.95, and expected heterozygosity (*He*) 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-

Table 3. Results of AMOVA performed with 27 SSR loci in 67 saffron ecotypes

Source of variation	Sum of squares	Variance components	Percentage variation
Among groups	59.638	1.68974	41.06066
Among populations within groups	33.456	0.26993	6.55925
Within populations	120.711	2.15556	52.38009
Total	213.806	4.11523	

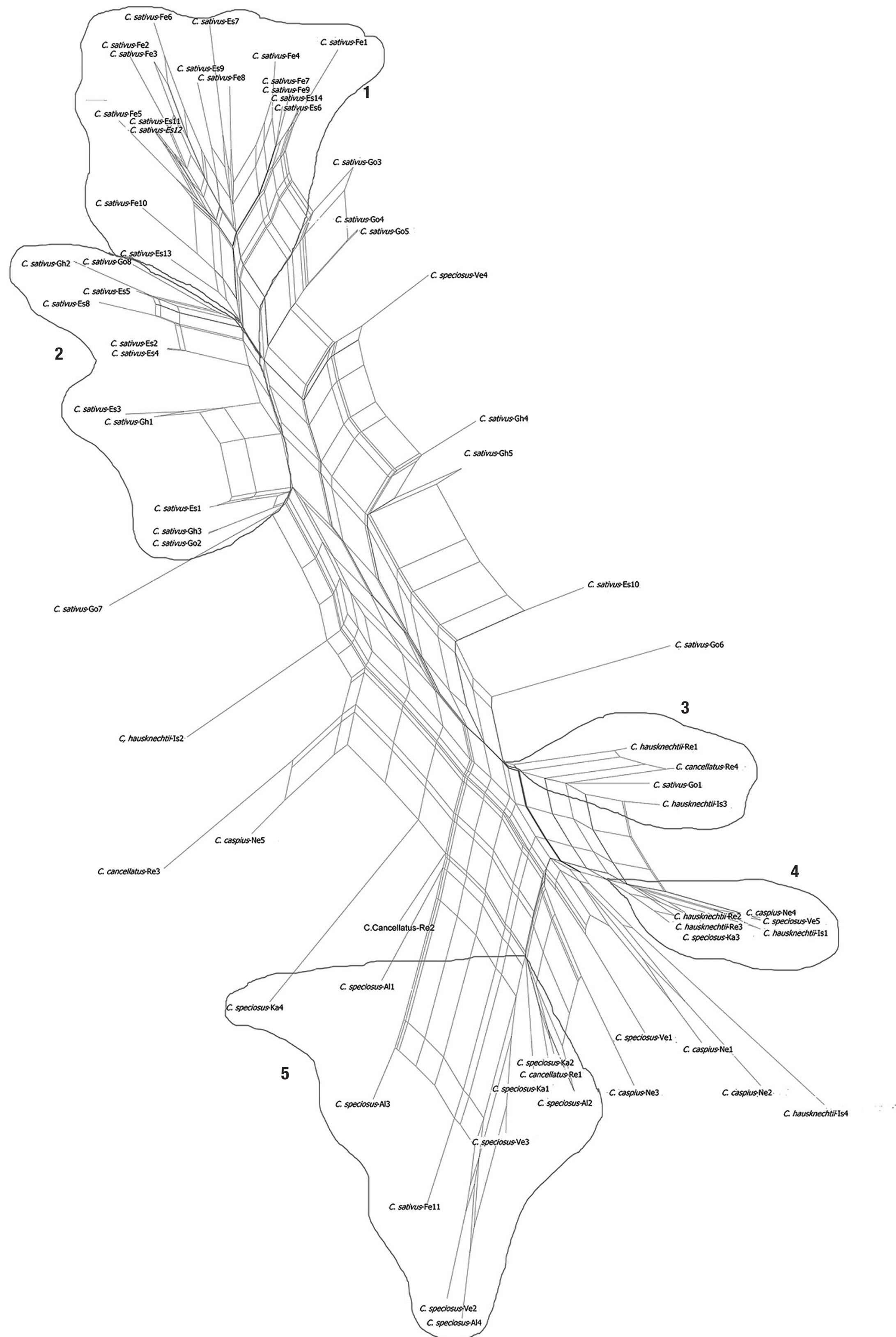

Figure 2. Phylogenetic relationships inferred based on SSR data using the distance-based Neighbor-Net method.

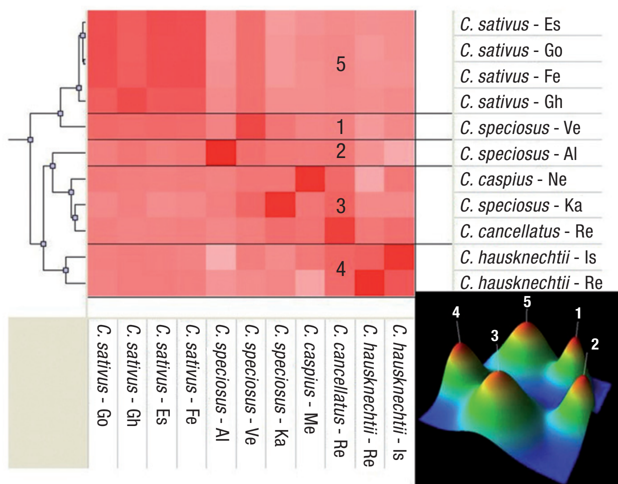

Figure 3. Agglomerative RB clustering methods of 67 individuals of Iranian saffron ecotypes (upper part). Matrix and 3D mountain visualizations generated by GCluto software (lower part). Each cluster is represented as a mountain with the following proportional parameters: height = internal similarities between its individuals, volume = number of objects in the cluster. The color of a peak represents the internal standard deviation of the cluster's objects. Red represents low deviation, whereas blue represents high deviation. The internal deviation is calculated by finding the average standard deviation of the pair-wise similarities between the cluster's objects (Rasmussen & Karypis, 2004).

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 (Alavi-Kia *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-

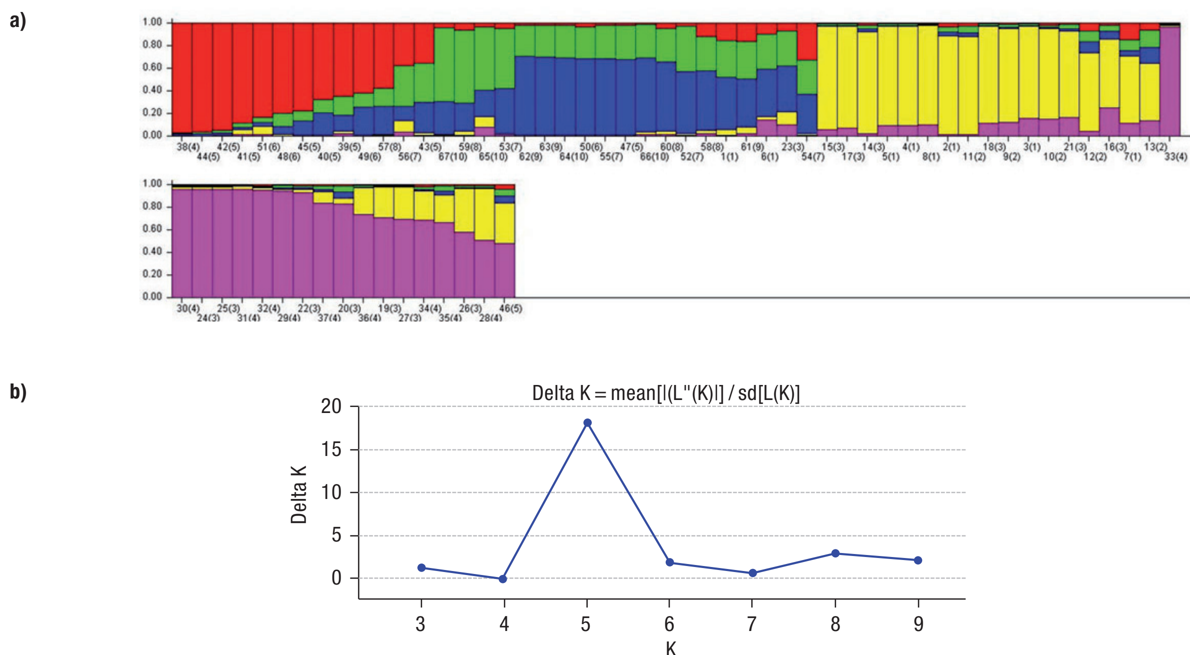

Figure 4. Population genetic structure of 67 saffron samples examined using STRUCTURE vers. 2.3.3 software. (a) Multiple colors in each vertical bar show admixed genetic constitution of each sample. Also, the number of *K* equals to the number of colors. (b) Each individual bar represents an ecotype based on the optimum *K*.

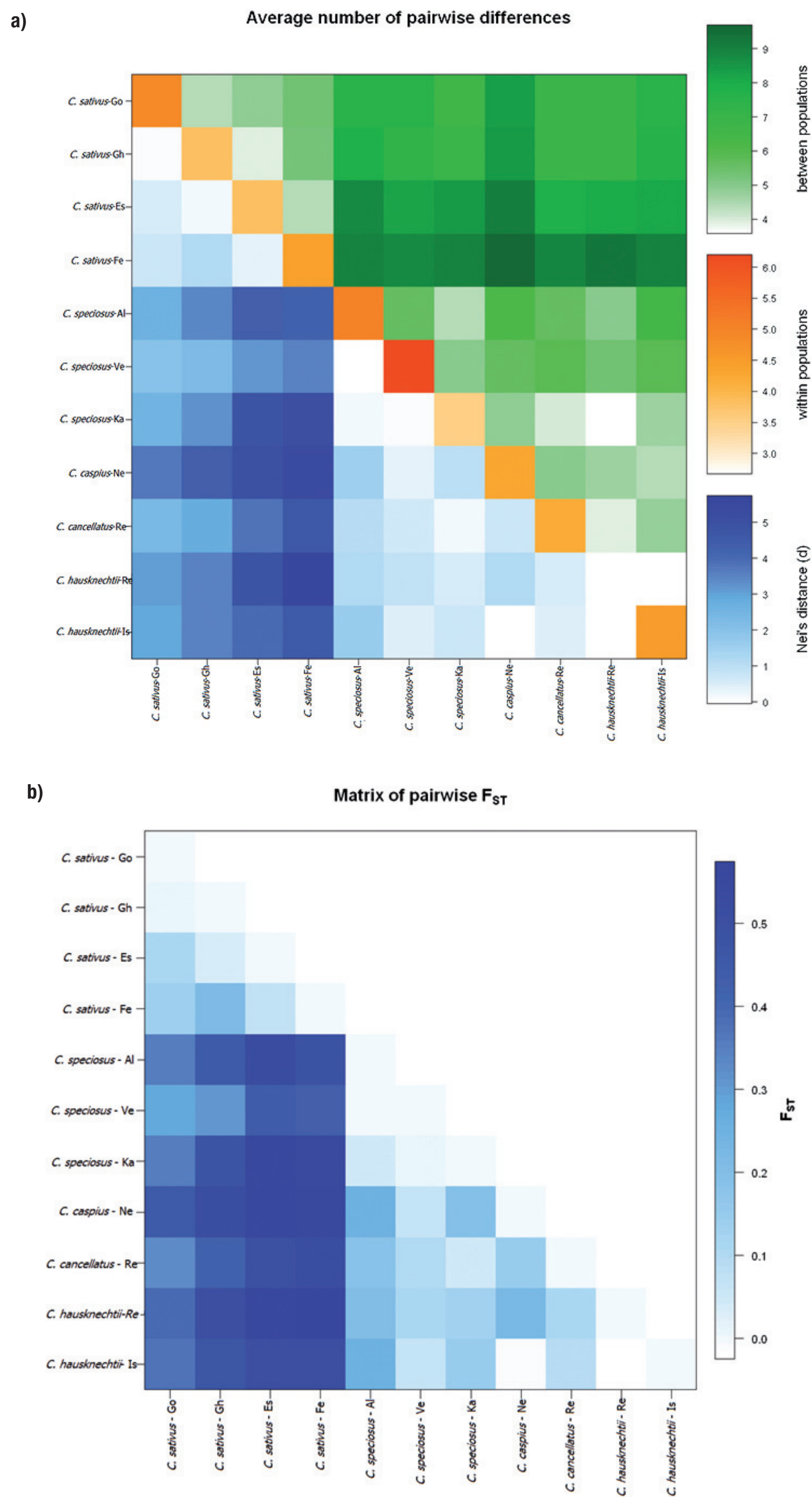

Figure 5. Average number of pairwise differences (a) and matrix of FST (b) shows the comparison among populations from ten geographical localities (see Fig. 1) based on microsatellite allele frequencies.

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.*

Acknowledgments

We thank to the many field assistants who helped with sample collection and to the Genomics Department at the Agricultural Biotechnology Research Institute of Iran (ABRII), for laboratory facilities and technical assistance.

References

Abdullaev FI, Espinosa-Aguirre JJ, 2004. Biomedical properties of saffron and its potential use in cancer therapy and chemoprevention trials. Cancer Detect Prev 28: 426-432.

Ahmad M, Zaffar G, Mir SD, Razvi SM, Rather MA, Mir MR, 2011. Saffron (*Crocus sativus* L.) strategies for enhancing productivity. Res J Medicinal Plant 5: 630-649.

Alavi-Kia SS, Mohammadi SA, Aharizad S, Moghaddam M, 2008. Analysis of genetic diversity and phylogenetic relationships in *Crocus* genus of Iran using inter retrotransposon amplified polymorphism. Biotechnol & Biotechnol Eq 22: 795-800.

Beiki AH, Keifi F, Mozafari J, 2010. Genetic differentiation of *Crocus* species by random amplified polymorphic DNA. Genet Eng Biotechnol J 18: 1-10.

Brighton CA, 1977. Cytology of *Crocus sativus* and its allies (*Iridaceae*). Plant Syst Evol 128: 137-157.

Bryant D, Moulton V, 2004. Neighbor-net: an agglomerative method for the construction of phylogenetic networks. Mol Biol Evol 21: 255-265.

Chryssanthi DG, Lamari FN, Iatrou G, Pylara A, Karamanos NK, Cordopatis P, 2007. Inhibition of breast cancer cell proliferation by style constituents of different *Crocus* species. Anticancer Res 27: 357-362.

Clark LV, Jasieniuk M, 2011. POLYSAT: an R package for polyploid microsatellite analysis. Mol Ecol Resour 11: 562-566.

Eckert CG, Samis KE, Lougheed C, 2008. Genetic variation across species geographical ranges: the central marginal hypothesis and beyond. Mol Ecol 17: 1170-1188.

Erol O, Kaya HB, Azik L, Tuna M, Can L, Tanyolac MB, 2014. The genus *Crocus*, series *Crocus* (*Iridaceae*) in Turkey and 2 East Aegean islands: a genetic approach. Turk J Biol 38: 48-62.

Evanno G, Regnaut S, Goudet J, 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14: 2611-2620.

Excoffier L, Lischer HEL, 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 10: 564-567.

Excoffier L, Smouse PE, Quattro JM, 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479-491.

Ferrence SC, Bendersky G, 2004. Therapy with saffron and the goddess at Thera. Perspect Biol Med 47: 199-226.

Grilli-Caiola M, Di-Somma D, Lauretti P, 2001. Comparative study of pollen and pistil in *Crocus sativus* L. (*Iridaceae*) and allied species. Annali Di Botanica 1: 93-103.

Huson DH, Bryant D, 2006. Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 23: 254-267.

Keify F, Beiki AH, 2012. Exploitation of random amplified polymorphic DNA (RAPD) and sequence-related amplified polymorphism (SRAP) markers for genetic diversity of saffron collection. J Med Plants Res 6: 2761-2768.

Mathew B, 1977. *Crocus sativus* and its allies (*Iridaceae*). Plant Syst Evol 128: 89-103.

Moretzsohn MC, Hopkins MS, Mitchell SE, Kresovich S, Valls JFM, Ferreira ME, 2004. Genetic diversity of peanut (*Arachis hypogaea* L.) and its wild relatives based on the analysis of hyper variable regions of the genome. BMC Plant Biol 4: 11.

Negbi M, Dagan B, Dror A, Basker D, 1989. Growth, flowering, vegetative reproduction and dormancy in the saffron *Crocus* (*C. sativus*). Israel J Bot 38: 95-113.

Nemati Z, Zeinalabedini M, Mardi M, Pirseyedi SM, Marashi SH, Khayam-Nekoui SM, 2012. Isolation and characterization of a first set of polymorphic microsatellite markers in saffron, *Crocus sativus* (*Iridaceae*). Am J Bot 99: 340e-343e.

Pritchard JK, Stephens M, Donnelly P, 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945-959.

Rasmussen M, Karypis G, 2004. gCLUTO: An interactive clustering, visualization, and analysis system. UMN-CS TR-04 21. http://glaros.dtc.umn.edu/gkhome/fetch/sw/gcluto/OLD/manual/index.html. [Accessed July 2004].

Rios JL, Recio MC, Giner RM, Manez S, 1996. An update review of saffron and its active constituents. Phytotherapy Res 10: 189-193.

Rubio-Moraga A, Castillo-López R, Gómez-Gómez L, Ahrazem O, 2009. Saffron is a monomorphic species as revealed by RAPD, ISSR and microsatellite analyses. BMC Res Notes 2: 189.

Rubio-Moraga A, Trapero-Mozos A, Gómez-Gómez L, Ahrazem O, 2010. Inter simple sequence repeat markers for molecular characterization of *Crocus cartwrightianus* cv. Albus. Ind Crop Prod 32: 147-151.

Siracusa L, Gresta F, Avola G, Albertini E, Raggi L, Marconi G, Lombardo GM, Ruberto G, 2012. Agronomic, chemical and genetic variability of saffron (*Crocus sativus* L.) of different origin by LC-UV-vis-DAD and AFLP analyses. Genet Resour Crop Ev 60: 711-721.

Weir BS, Cockerham C, 1984. Estimating *F* statistic for the analysis of population structure. Evolution 38: 1358-1370.

Winterhalter P, Straubinger M, 2000. Saffron renewed interest in an ancient spice. Food Rev Int 16: 39-59.