TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2014) 38: 48-62
© TÜBİTAK
doi:10.3906/biy-1305-14

The genus *Crocus*, series *Crocus* (Iridaceae) in Turkey and 2 East Aegean islands: a genetic approach

Osman EROL[1], Hilal Betül KAYA[2], Levent ŞIK[3], Metin TUNA[4], Levent CAN[5,*], Muhammed Bahattin TANYOLAÇ[2]

[1]Department of Botany, Faculty of Science, İstanbul University, İstanbul, Turkey
[2]Department of Bioengineering, Faculty of Engineering, Ege University, Bornova, İzmir, Turkey
[3]Department of Biology, Faculty of Arts and Sciences, Celal Bayar University, Manisa, Turkey
[4]Department of Field Crops, Faculty of Agriculture, Namık Kemal University, Tekirdağ, Turkey
[5]Department of Biology, Faculty of Arts and Sciences, Namık Kemal University, Tekirdağ, Turkey

Received: 06.05.2013 • **Accepted:** 02.08.2013 • **Published Online:** 02.01.2014 • **Printed:** 24.01.2014

Abstract: In this study, a total of 26 *Crocus* specimens from different locations across Turkey and 2 East Aegean islands (Chios and Samos) were analyzed using 12 amplified fragment length polymorphism (AFLP) primer combinations to obtain information on genetic diversity, population structure, and genetic relationships. A total of 369 polymorphic AFLP bands were generated and scored as binary data. Genetic similarities were determined. Cluster analysis revealed 4 major groups among the 26 genotypes examined in this study. The nuclear DNA contents (2C) of the 26 *Crocus* specimens were found to range from 5.08 pg in *C. asumaniae* to 9.75 pg in *C. sativus*. Polymorphic information content (PIC) values were used to examine the capacity of the various primer pairs to amplify polymorphisms in the *Crocus* specimens. The PIC values ranged from 0.218 (M-CAA/E-AGC) to 0.512 (M-CAT/E-AAG) and showed an average of 0.34. In sum, we herein used AFLP analysis to identify a high level of polymorphism among *Crocus* specimens collected from various locations in Turkey and Greece, and our structural analysis yielded 2 reconstructed populations. These findings provide new insight into the relationships among different *Crocus* genotypes and show that AFLP analysis can be useful for *Crocus* diversity studies.

Key words: Amplified fragment length polymorphism, *Crocus*, genetic diversity, genetic structure, nuclear DNA content

1. Introduction

The genus *Crocus* L. belongs to the large family Iridaceae and is a systematically problematic genus. In the Old World, about 100 species (Harpke et al., 2013) are distributed between 10°W and 80°E and between 30°N and 50°N (Mathew, 1982). Phytogeographically, most *Crocus* species belong to the Mediterranean floristic region, with an additional range into the Irano-Turanian phytochorion. The species of this genus occur in climates characterized by a chilly or cold winter, rainy spring and autumn, and hot and dry summer. The developmental activity of the plant can be observed from autumn to spring; it survives the summer heat beneath the soil with its compact corm underground. Numerous species begin to grow their aerial parts during the autumn rains and flower afterwards. Some flower simultaneous with leaf growth or soon thereafter, while others flower in the spring when it is warmer.

Based on the studies of Mathew (1982) and Mathew et al. (2009), the autumn-flowering *Crocus sativus* L., which produces the most expensive relict agricultural product in the world (saffron), was gathered with its relatives in the series *Crocus* (Table 1). That work was prepared according to morphological differences, as genetic tools were not commonly utilized back then. However, morphological characteristics can be affected by environmental factors acting during the developmental stages of the plant (Jonah et al., 2011), and the use of morphological characteristics in diversity studies could lead to misclassification (Joshi et al., 2011). Furthermore, numerous new taxa have been introduced in the botanical literature since the extensive work of Mathew (1982), and his subspecies system is no longer considered valid (Kerndorff and Pasche, 2011; Kerndorff et al., 2011).

Molecular markers such as DNA (Lee, 1995) and isozymes (Winter and Kahl, 1995) are not affected by developmental processes or environmental influences and are used for determination of genetic diversity (Hamza et al., 2012; Poyraz et al., 2012; Sönmezoğlu et al., 2012; Taşkın et al., 2012; Türktaş et al., 2012; Zhang et al., 2012). They can be used to characterize organisms at the genomic level, yielding resolution that cannot be acquired by conventional systematic studies (Jonah et al., 2011).

* Correspondence: lcan@nku.edu.tr

Table 1. Taxa belonging to series *Crocus* and their countries of distribution.

	Taxon	Distribution (country)
1	*Crocus asumaniae* B.Mathew & T.Baytop	Turkey
2	*Crocus cartwrightianus* Herb.	Greece
3	*Crocus sativus* L.	Spain, Greece, Italy, Morocco, Turkey, Iran, Pakistan
4	*Crocus hadriaticus* Herb. subsp. *hadriaticus*	Greece
5	*Crocus hadriaticus* subsp. *parnassicus* (B.Mathew) B.Mathew	Greece
6	*Crocus hadriaticus* subsp. *parnonicus* B.Mathew	Greece
7	*Crocus moabiticus* Bornm. & Dinsm. ex Bornm.	Jordan, Israel
8	*Crocus mathewii* Kernd. & Pasche	Turkey
9	*Crocus naqabensis* Al-Eisawi	Jordan, Israel
10	*Crocus oreocreticus* B.L.Burtt	Greece
11	*Crocus pallasii* Goldb. subsp. *pallasii*	Bulgaria, Romania, Macedonia, Ukraine, Greece, Turkey, Syria, Lebanon, Israel
12	*Crocus pallasii* subsp. *dispathaceus* (Bowles) B.Mathew	Turkey, Syria, Lebanon
13	*Crocus pallasii* subsp. *haussknechtii* (Boiss. & Reut. ex Maw) B.Mathew	Iran, Iraq, Israel
14	*Crocus pallasii* subsp. *turcicus* B.Mathew	Turkey, Iraq, Syria, Lebanon
15	*Crocus thomasii* Ten.	Italy, Croatia

As such, molecular markers are an effective method for obtaining information on genetic diversity and population structure (Odong et al., 2011). This can be extremely valuable to the preservation of wild species, as loss of genetic variability may reduce survival chances in the wild (Swanson, 1996). Studies on population genetics have attracted much attention because genetic diversity and variance are particularly important for the sustainability of species. Moreover, investigations of genetic diversity and population structure can provide important information for the management of genetic resources and the conservation of biodiversity in plants (Manel et al., 2003; Odong et al., 2011). In terms of methods, STRUCTURE is a Bayesian model-based algorithm that is widely used to cluster genetic data (Pritchard et al., 2000; Hubisz et al., 2009). For K ancestral populations, and assuming Hardy–Weinberg and linkage equilibrium within clusters, STRUCTURE estimates the allele frequencies in each cluster and the population membership for every individual (Pritchard et al., 2000). In the admixture model, it estimates admixture proportions for each individual and uses the Markov chain Monte Carlo approach to integrate the information over the parameter space and make cluster assignments (Pritchard et al., 2000).

Although molecular studies have been increasingly used to examine the phylogeny of living organisms, they have only recently been applied to the genus *Crocus* (Petersen et al., 2008). DNA markers have been used to characterize germplasm collections, inform breeding programs, and facilitate genetic diversity studies and taxonomic analysis. The utilized methods include inter-retrotransposon amplified polymorphism (IRAP) (Alavi-Kia et al., 2008), random amplified polymorphic DNA (RAPD) (Grilli Caiola et al., 2004; Beiki et al., 2010; Rubio-Moraga et al., 2010), amplified fragment length polymorphism (AFLP) (Zubor et al., 2004; Erol et al., 2011; Nazzal et al., 2011), intersimple sequence repeat (ISSR) (Rubio-Moraga et al., 2010), and simple sequence repeat (SSR) (Rubio-Moraga et al., 2010; Nemati et al., 2012) analyses. Among these molecular markers, AFLPs were found to show high levels of polymorphism per primer pair and yield high resolution and reproducibility (Meudt and Clarke, 2007). This technique for typing genomic DNA is based on the selective PCR amplification of DNA restriction fragments from total digests of genomic DNA (Vos et al., 1995; Powell et al., 1996).

Nuclear DNA content is a key karyological feature for systematic and evolutionary assessments in biology, biodiversity, and molecular investigations, as the genome size has many important practical applications (Bennett and Leitch, 1995). Flow cytometry (FCM) is considered one of the most rapid and exact techniques for predicting nuclear DNA content in plants (Doležel et al., 1989), and it also has been used to determine nuclear DNA content

of *Crocus* species in one case. Brandizzi and Grilli Caiola (1998) estimated the 2C nuclear DNA content of saffron (*Crocus sativus*, 2n = 3x = 24) as 11.35 ± 0.04 pg. Ignoring polyploidy and a few other traps, this value is typically constant within a species.

In the present study, we used AFLP analysis plus FCM to investigate the genetic diversity and population structuring of the taxa of *Crocus*, series *Crocus* distributed in Turkey and the East Aegean islands, i.e. from the center of species diversity of the genus (Harpke et al., 2013) (*Crocus pallasii* Goldb. subsp. *pallasii*, *Crocus pallasii* subsp. *turcicus* B.Mathew, *Crocus pallasii* subsp. *dispathaceus* (Bowles) B.Mathew, *Crocus sativus* L., *Crocus asumaniae* B.Mathew & T.Baytop, and *Crocus mathewii* Kernd. & Pasche). Our results demonstrated that AFLP provides a highly efficient tool for determining genetic variations among *Crocus* genotypes. Accurate analysis of the genetic diversity and structure among the studied genotypes could facilitate the establishment of management practices to preserve this species, and our investigation provides essential information for future studies of the genetic relationship among *Crocus* taxa.

2. Materials and methods

2.1 Taxon sampling

Taxa were sampled as widely as possible from Turkey and the East Aegean islands. Specimens were collected from the field between 2008 and 2011 (Figure 1) and grown in pots at the İstanbul University Alfred Heilbronn Botanical Garden (İstanbul, Turkey). The pots were buried in coarse sand to ensure a suitable moisture level. Morphological observations were based on materials both cultivated at the İstanbul University Alfred Heilbronn Botanical Garden and obtained from the field. Molecular data were obtained from cultivated material. The materials included in this study are listed in Table 2, with localities shown on the map in Figure 1. Intraspecific categories that were morphologically ambiguous were ignored during the field studies, allowing us to make objective observations and avoid prejudgment.

2.2. Leaf anatomy

Leaves were cut from specimens and put into a 70% ethyl alcohol and 5% glycerin solution. Cross-sections were obtained by sectioning the leaves from the tip to the midpoint (15–20 mm from the tip), using either a razor blade or an ice microtome. The cross-sections were dyed with SARTUR solution (Çelebioğlu and Baytop, 1949), and slides were made with Plastic UV Mounting Media (PolyScience) and 1 to 2 drops of xylol and were then placed under a UV polymerization lamp.

2.3. DNA extraction

Genomic DNA was extracted from the leaves, previously frozen in liquid nitrogen, using the CTAB protocol described by Doyle and Doyle (1990). The extracted DNA

Figure 1. Geographical distribution of the obtained specimens of series *Crocus*. The numbers correspond to the specimen numbers in Table 2 (Google Inc., 2012).

Table 2. List of studied specimens with specimen numbers, origin, total DNA content, and membership proportion for each pre-defined population.

No.	Specimen no.	Taxa	District	DNA content (pg/2C)	P* ≥70%	Cluster1	Cluster2
1	SB60	Undefined taxon	Antalya	8.89 pg	1	0.721	0.279
2	SC1	*C. pallasii* subsp. *pallasii*	Muğla, Fethiye	5.81 pg	1	0.939	0.061
3	SC2	*C. pallasii* subsp. *pallasii*	Muğla, Fethiye	5.95 pg	Admixture	0.345	0.655
4	SC4	*C. mathewii*	Muğla, Fethiye	6.17 pg	1	0.957	0.043
5	SC5	*C. pallasii* subsp. *pallasii*	Muğla	5.80 pg	Admixture	0.302	0.698
6	SC6	*C. pallasii* subsp. *pallasii*	Muğla	5.66 pg	2	0.009	0.991
7	SC7	*C. pallasii* subsp. *pallasii*	Muğla	5.98 pg	2	0.222	0.778
8	SC8	*C. pallasii* subsp. *pallasii*	Muğla	5.89 pg	1	0.989	0.011
9	SC9	*C. mathewii*	Antalya	5.81 pg	Admixture	0.674	0.326
10	SC10	*C. pallasii* subsp. *pallasii*	İzmir	5.45 pg	Admixture	0.654	0.346
11	SC12	*C. pallasii* subsp. *pallasii*	Denizli	5.82 pg	1	0.977	0.023
12	SC17	*C. pallasii* subsp. *pallasii*	Konya	5.45 pg	2	0.3	0.7
13	SC18	*C. pallasii* subsp. *pallasii*	Konya	6.41 pg	Admixture	0.503	0.497
14	SC20	*C. pallasii* subsp. d*ispathaceus*	Mersin	5.59 pg	Admixture	0.629	0.371
15	SC21	*C. pallasii* subsp. *dispathaceus*	Mersin	6.75 pg	1	0.944	0.056
16	SC22	*C. pallasii* subsp. *turcicus*	Gaziantep	8.47 pg	1	0.72	0.28
17	SC42	*C. pallasii* subsp. *pallasii*	Konya	5.73 pg	Admixture	0.428	0.572
18	SC45	*C. asumaniae*	Antalya	5.17 pg	2	0.015	0.985
19	SC47	*C. asumaniae*	Antalya, İbradı	5.08 pg	1	0.983	0.017
20	CJGR-001	*C. pallasii* subsp. *pallasii*	Chios	5.87 pg	2	0.013	0.987
21	CJGR-047	*C. pallasii* subsp. *pallasii*	Chios	5.89 pg	1	0.989	0.011
22	CJGR-060	*C. pallasii* subsp. *pallasii*	Chios	6.25 pg	2	0.116	0.884
23	CJGR-072	*C. pallasii* subsp. *pallasii*	Samos	6.02 pg	2	0.128	0.872
24	CJGR-068	*C. pallasii* subsp. *pallasii*	Samos	5.69 pg	1	0.94	0.06
25	DDGL	*C. pallasii* subsp. *pallasii*	Isparta	6.57 pg	Admixture	0.671	0.329
26	CSTV	*C. sativus*	Cultivated material from Kastamonu	9.75 pg	Admixture	0.666	0.334

was visualized by 1% agarose gel electrophoresis, and its concentration and purity were spectrophotometrically quantified using an ND-1000 (NanoDrop, Thermo, Inc.). The DNA was diluted to a working concentration of 40 ng/µL.

2.4. AFLP analysis

AFLP analysis (Vos et al., 1995) was performed using a commercially available kit from Li-COR Biosciences (Lincoln, NE, USA). Briefly, 200 ng of genomic DNA was digested with *Eco*RI and *Mse*I, ligated with adapters of these restriction enzymes, and subjected to preamplification using primers based on the adapter sequences. The diluted (40-fold) preamplified products were used as a template for selective amplifications. A total of 12 primer combinations of *Eco*RI and *Mse*I with 3-nucleotide extensions at their 3'-ends were used (Table 3). The *Eco*RI primers were 5'-labeled with infrared dyes (IRDye 700 or IRDye 800, Li-COR). PCR was conducted on a PTC-220 Dyad Thermal Cycler (MJ Research Inc., Waltham, MA, USA). The amplification products were subjected to 8% polyacrylamide gel electrophoresis on a 4300s DNA Analyzer system (Li-COR). Clear, unambiguous bands were manually scored as present or absent. All loci were scored twice, independently, to minimize scoring errors (Figure 2).

2.5. Data analysis

For genetic diversity analysis, polymorphic bands from each sample were recorded manually. A band was considered polymorphic if it appeared in at least 1 genotype while being absent from at least 1 other genotype. Each

Table 3. Total polymorphic bands and polymorphic information content (PIC) obtained from the 12 AFLP primer combinations.

Combination	Primers	Number of polymorphic bands	PIC
1	M-CTA/E-AAG	43	0.282
2	M-CTA/E-AGG	44	0.301
3	M-CAC/E-ACA	55	0.455
4	M-CAC/E-ACG	34	0.347
5	M-CAA/E-AAG	48	0.442
6	M-CAA/E-ACG	7	0.253
7	M-CAA/E-AGA	29	0.263
8	M-CAA/E-AGC	18	0.218
9	M-CAT/E-ACA	30	0.296
10	M-CAT/E-ACT	17	0.319
11	M-CAT/E-AAG	22	0.512
12	M-CAT/E-AGC	22	0.430

band was scored as '1' if present and '0' if absent. Similarity matrices were produced using the Jaccard similarity coefficient (Jaccard, 1908) and processed with the SAHN computational module of NTSYS-pc version 2.2 (Rohlf, 2005). A dendrogram was constructed according to the unweighted pair group method with arithmetic means algorithm (UPGMA) (Figure 3). The discriminating power of the derived markers was assessed using the polymorphism information content (PIC), calculated as follows:

$$PIC=1-\sum_{i=1}^{n}P_i^2$$

where PIC is the frequency of the *i*th allele in the *j*th population for each locus (Botstein et al., 1980; Anderson et al., 1993; Muthusamy et al., 2008; Uzun et al., 2009). Principal coordinate analysis (PCA) based on the genetic similarity matrix was used to visualize the genetic relationships among the 26 *Crocus* genotypes using AFLP

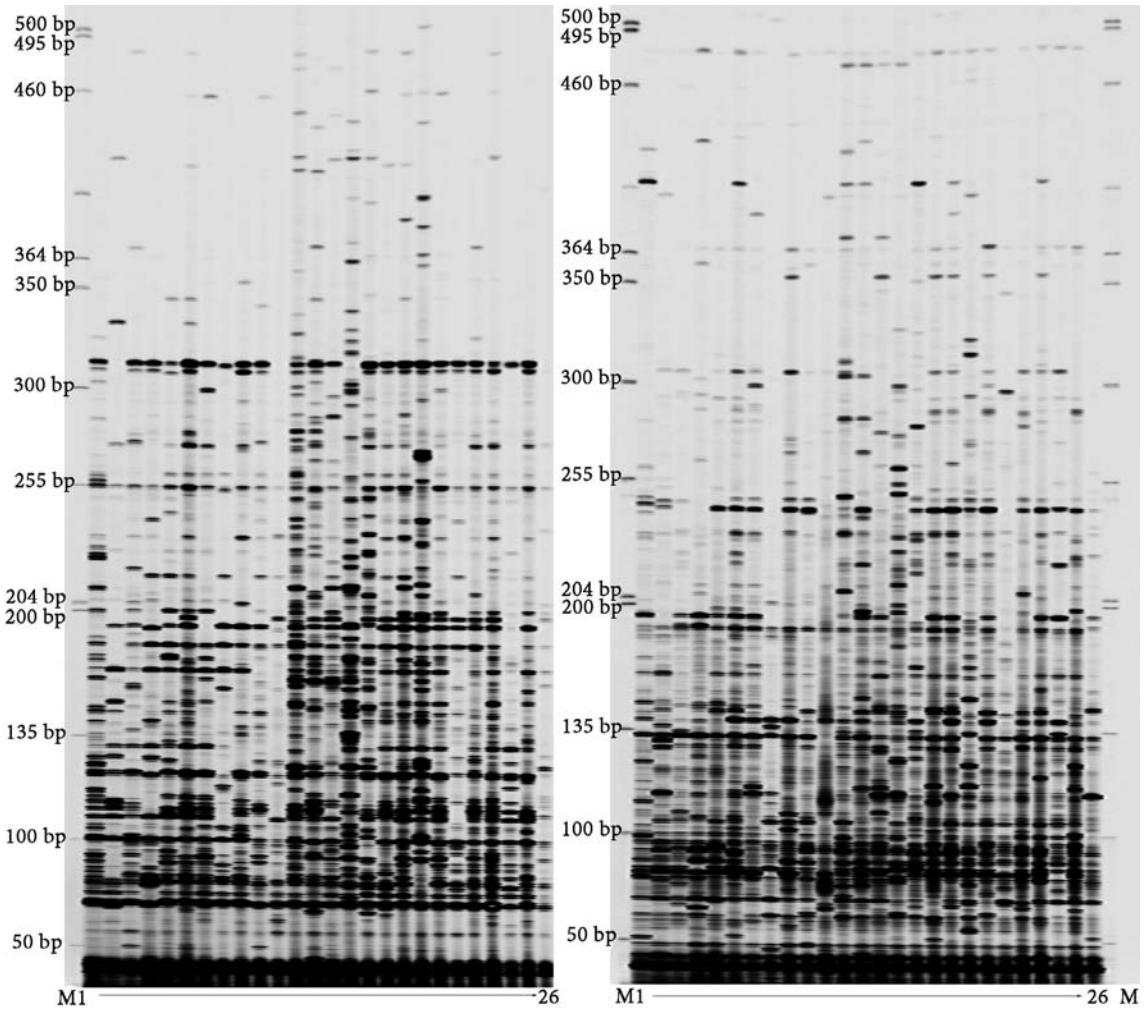

Figure 2. AFLP profiles showing the genetic polymorphisms among 26 *Crocus* genotypes as detected using the selective primer combinations M-CAT/E-ACA (a) and M-CAT/E-ACT (b).

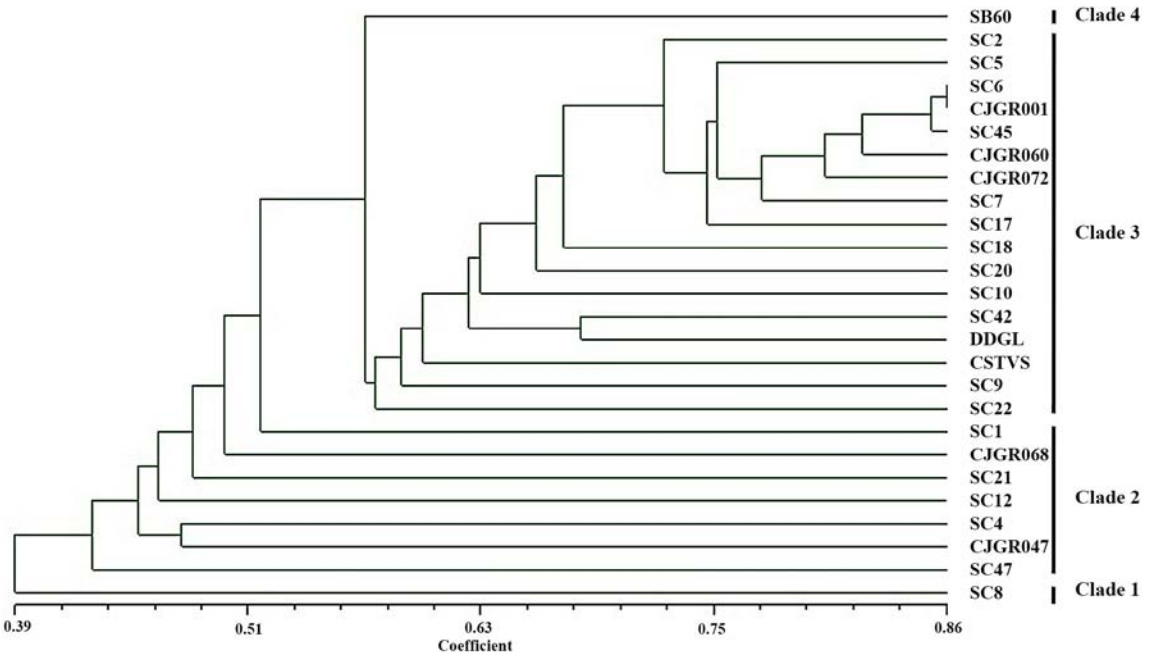

Figure 3. Dendrogram showing the genetic relationships among the 26 *Crocus* specimens based on AFLP data, UPGMA clustering, and Jaccard's coefficients.

data and NTSYS version 2.2 (Rohlf, 2005). PCA was carried out using the EIGEN module.

To investigate population structure in *Crocus*, we used a model-based approach implemented in the STRUCTURE 2.3.4 software (Pritchard et al., 2000), which tests hypothetical numbers of subpopulations to infer the number of populations into which the analyzed genotypes can be divided. We ran 5 independent STRUCTURE analyses with the number of subgroups (K) ranging from 1 to 10. Each run was implemented with a burn-in period of 10,000 steps followed by 100,000 Monte Carlo Markov chain replicates (Hubisz et al., 2009). The population structure was analyzed assuming admixture in the population and using a correlated allele frequency model. Evanno's method (Evanno et al., 2005) was used to identify the appropriate number of clusters via the ad hoc statistic, Δk, which is based on the second-order rate of change of the likelihood function with respect to successive values of K. Based on the STRUCTURE output file, the number of true clusters in the data (K) was determined using Structure Harvester (Earl and von Holdt, 2012), which identifies the optimal K based on the ΔK and the posterior probability of the data for a given K (Evanno et al., 2005).

2.6. Total DNA content

The total DNA content was determined as described by Arumuganathan and Earle (1991). The fluorescence intensities of the stained nuclei were measured using a CYTOMICS FC 500 flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA). The values for nuclear DNA content were estimated by comparing the fluorescence intensities of the nuclei in the test population with those of an appropriate internal DNA standard run in parallel. We used vetch (*Vicia sativa* L.) as the internal standard; it is a diploid (2x = 14) species that has a 1C DNA complement of 1.65 pg per nucleus. For FCM analysis, 50 mg of fresh leaf tissue was excised from adult pot-grown plants, placed on ice in a sterile plastic petri dish, and mixed with 20 mg of fresh leaf tissue from vetch (standard). The tissues were chopped into 0.25- to 1-mm segments in 1 mL of solution A [24 mL of $MgSO_4$ buffer (ice-cold), 25 mg of dithiothreitol, 500 µL of propidium iodide stock (5.0 mg of propidium iodide in 1.0 mL of double-distilled H_2O), and 625 µL of Triton X-100 stock (1.0 g of Triton X-100 in 10 mL of double-distilled H_2O)]. The solution and tissue were filtered through a 30-µm nylon mesh into a microcentrifuge tube, and the tube was centrifuged at high speed (13,000 rpm) for about 15–20 s. The supernatant was discarded, the pellet was resuspended in 400 µL of solution B (7.5 mL of solution A containing 17.5 of µL DNase-free RNase) and incubated for 20 min at 37 °C, and FCM analysis was performed. Samples stained with propidium iodide were excited with a 15-mW argon ion laser at 488 nm. Red propidium iodide fluorescence area signals (FL2A) from nuclei were collected in the FL2 channel. The mean DNA content per sample (2C) was based on analysis of 1000 nuclei per sample.

3. Results

3.1. Morphological observations

While collecting the taxa of *Crocus* series *Crocus* in Turkey, we paid particular attention to the southwestern

part of Anatolia. According to recent investigations, the southwestern part of Asia Minor is an important diversity center of this group. The taxa of series *Crocus* collected from the Muğla-Fethiye-Antalya "triangle" showed a particularly high degree of morphological variation.

According to Mathew (1982), the taxa belonging to the *Crocus pallasii* complex are distinguished by "neck" characteristics formed by overlaps of the corm tunics and cataphylls. However, our field studies revealed that this character is not as stable as suggested. The published distinction of *C. pallasii* subsp. *pallasii* and *C. pallasii* subsp. *turcicus* depends largely on this characteristic; *turcicus* has a longer neck (up to 6 cm), whereas *pallasii* has a poorly developed neck (<2 cm) (Mathew, 1984). However, we observed quite long necks in specimens SC20 and SC21 (*C. pallasii* subsp. *dispathaceus*).

Crocus mathewii, which has distinctive white flowers and a sulfide-colored neck, is one of the easily distinguishable plants belonging to this series. This flower color seems to be stable for the populations at the type locality in Antalya and also in Muğla, but the specimens in the locality where SB60 was collected showed variations in this characteristic (some of the plants had the typical sulfide-colored neck, while others did not). Some of the plants did not have the character but seemed more likely to be *C. pallasii* subsp. *pallasii* specimens. SB60 also differed from its relative in terms of tepal shape, style, and color.

Tepal shape and size are important morphological characteristics. The size seems to be stable for *Crocus pallasii* subsp. *dispathaceus* (segments are 4–8 mm wide), but the tepal color of this taxon appears to differ. The Turkish populations had deep purple flowers, while the Syrian populations had brownish flowers. In the future, this difference should be clarified by a detailed study of both populations.

The complexity and convergence of the morphological characteristics of the observed taxa, both in the field and in herbaria, emphasizes the need for a molecular-level phylogenetic analysis.

3.2. Anatomy

The leaf anatomy of the collected specimens was examined in terms of their overall aspect, arm positions, the shape of carina, the ridges underneath the leaf, the mesophyll, and the shape of the epidermal cells (Rudall and Mathew, 1990; Erol and Küçüker, 2007). Only specimen SC8 (*Crocus pallasii* subsp. *pallasii*) showed a distinctive and unexpected anatomical characteristic: the cross-sections of its leaves had distinct ribs with simple hairs (Figure 4). This structure (ribs with hairs) is new for the genus. We did not observe any other important morphological differences between SC8 and the other specimens of *C. pallasii* subsp. *pallasii*.

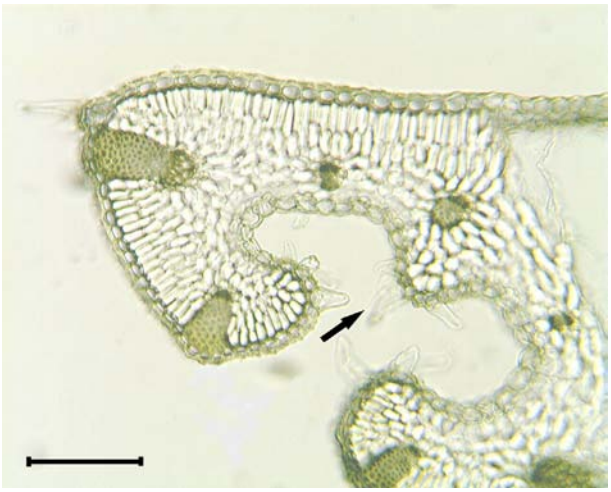

Figure 4. A leaf cross-section of *Crocus pallasii* (SC8). Arrow shows the rib and hairs in the stomatal region (black bar = 100 μm).

3.3. Marker polymorphisms

Crocus taxa were analyzed using 12 AFLP primer combinations, and all 12 produced reproducible polymorphic banding patterns. Clear and unambiguous bands of 50 to 500 bp were considered usable, and 369 polymorphic fragments were obtained. The number of bands generated per primer pair varied from 7 (M-CAA/E-ACG) to 55 (M-CAA/E-ACA) (Table 3), with an average of 30.8. AFLP profiles from representative gels are shown in Figure 3. In terms of discrimination power, the AFLP markers had average PIC values ranging from 0.218 to 0.512, with an overall average of 0.34 per primer (Table 3).

3.4. Genetic structure and diversity

The STRUCTURE program was used to determine the population structure among the *Crocus* genotypes (Figure 5). To calculate the most appropriate K value for the 26 genotypes, we utilized an ad hoc statistical analysis based on the second-order rate of change of the likelihood function with respect to K (ΔK), as described by Evanno et al. (2005) and applied by the Structure Harvester v. 0.6.92 software program (Earl and von Holt, 2012). There was a clear peak in the value of ΔK at K = 2.

A clustering bar plot for K = 2 is shown in Figure 5. At K = 2, all 26 *Crocus* genotypes were divided into 2 groups. No clear geographic structure was detected using this approach.

Of the 26 *Crocus* genotypes 17 (65.4%) shared >70% membership with 1 of 2 clusters and were classified as members of that cluster, whereas 9 genotypes (34.7%) were categorized as admixture forms with varying levels of membership shared between the 2 clusters. Cluster 1 consists of 10 genotypes (1 unpublished genotype from Antalya; 3 from Muğla; and 1 each from Denizli, Mersin,

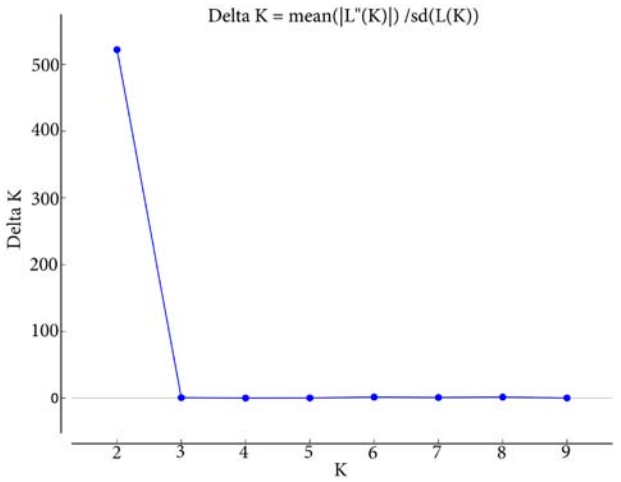

Figure 5. STRUCTURE-based estimate of the number of populations for K (ranging from 1 to 10) by ΔK values.

Gaziantep, Antalya, Chios, and Samos). Cluster 2 consists of 7 genotypes (2 from Muğla; 2 from Chios; and 1 each from Konya, Antalya, and Samos). The remaining 9 genotypes (2 from Konya; 2 from Muğla; and 1 each from Antalya, İzmir, Mersin, Isparta, and Kastamonu) were classified as admixtures (Figure 6; Table 2).

The genetic similarity coefficients among the 26 *Crocus* specimens varied widely, between 0.29 and 0.86 (Table 4). The maximum similarity of 86% was observed between genotypes SC6 and CJGR-001, while the lowest similarity of 3% was observed between genotypes SC8 and SC47. To evaluate the phylogenetic relationships among the 26 *Crocus* specimens, we used the UPGMA method to construct a dendrogram based on Jaccard's coefficient of standard genetic similarity (Figure 6). The dendrogram distributed the specimens into 4 clades. The lowest genetic similarity was recorded between SC8 from clade 1 and SC47 from clade 2, while the highest genetic similarity was observed between SC6 and CJGR-001, both of which were placed in clade 3.

PCA based on the AFLP data distributed the populations into 4 distinct groups (Figure 7), confirming the results of our cluster analysis.

3.5. Nuclear DNA content

FCM was used to measure the fluorescence of isolated, propidium iodide-stained intact nuclei, and we calculated the nuclear DNA content with respect to that of an internal standard with an established genome size. The 2C DNA contents of the analyzed genotypes ranged from 5.08 pg in *Crocus asumaniae* to 9.75 pg in *C. sativus*.

4. Discussion

Series *Crocus* harbors morphologically problematic taxa, particularly the subspecies of *Crocus pallasii*. For example, *Crocus pallasii* subsp. *turcicus* is distinguished by the "tunic neck" of its cataphylls, but this characteristic can also be seen in populations of *Crocus pallasii* subsp. *pallasii* and *Crocus pallasii* subsp. *dispathaceus* (Figure 8.). The neck characteristic of *Crocus pallasii* subsp. *turcicus* is thus useless in the field and in herbaria. However, nuclear DNA content and chromosome number can be used to distinguish these subspecies.

This study sought to measure genetic diversity and investigated the genetic structure among 26 *Crocus* genotypes using AFLP markers. Given its low cost and high efficiency, the PCR-based AFLP genome fingerprinting technique has become a common molecular tool for the assessment of genetic variation among plant populations originating from different geographical sites (Mueller and Wolfenbarger, 1999).

The 12 primer pairs generated clear AFLP profiles for the 26 *Crocus* genotypes. The amplified fragments ranged from 50 to 500 bp, and a total of 369 scorable fragments were identified. Seven to 55 polymorphic bands were amplified per primer pair, with an average of 30.8 polymorphic bands per pair. Erol et al. (2011) observed 981 polymorphic fragments with an average of 44.6 polymorphic loci per primer in their AFLP analysis, which is relatively consistent with our present results. However, Nazzal et al.

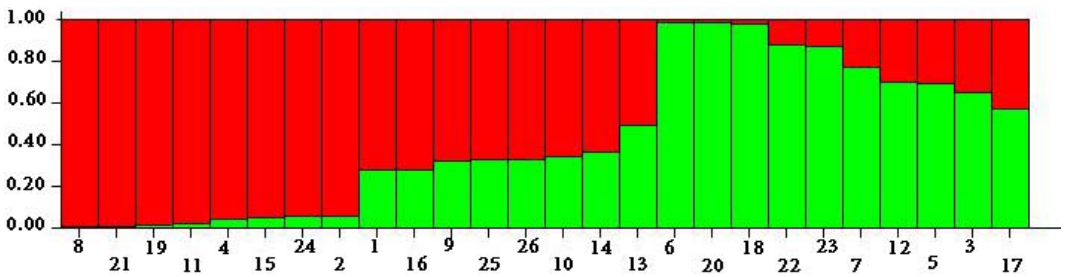

Figure 6. Two subgroups inferred from the STRUCTURE analysis. The vertical coordinate of each subgroup indicates the membership coefficient for each individual. Bars with 2 colors represent genotypes that show admixture.

Table 4. Genetic similarities among 26 *Crocus* genotypes, calculated based on Jaccard's coefficient. The numbers represent the taxa listed in the first column of Table 2.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	1.00																									
2	0.43	1.00																								
3	0.59	0.51	1.00																							
4	0.43	0.35	0.51	1.00																						
5	0.56	0.55	0.70	0.48	1.00																					
6	0.64	0.54	0.75	0.50	0.77	1.00																				
7	0.56	0.55	0.74	0.52	0.72	0.79	1.00																			
8	0.38	0.37	0.41	0.38	0.45	0.44	0.42	1.00																		
9	0.48	0.48	0.61	0.48	0.61	0.63	0.63	0.37	1.00																	
10	0.56	0.49	0.59	0.43	0.64	0.64	0.62	0.40	0.51	1.00																
11	0.43	0.46	0.48	0.39	0.53	0.49	0.50	0.33	0.44	0.48	1.00															
12	0.58	0.52	0.67	0.47	0.73	0.77	0.70	0.45	0.57	0.64	0.49	1.00														
13	0.57	0.54	0.62	0.45	0.65	0.70	0.62	0.38	0.56	0.60	0.44	0.68	1.00													
14	0.58	0.52	0.64	0.49	0.65	0.65	0.64	0.39	0.56	0.60	0.53	0.65	0.64	1.00												
15	0.42	0.43	0.46	0.39	0.50	0.53	0.51	0.36	0.45	0.47	0.38	0.57	0.52	0.48	1.00											
16	0.56	0.46	0.59	0.45	0.56	0.62	0.63	0.35	0.47	0.56	0.39	0.60	0.58	0.58	0.44	1.00										
17	0.54	0.54	0.61	0.47	0.63	0.72	0.64	0.40	0.58	0.60	0.44	0.62	0.59	0.60	0.44	0.50	1.00									
18	0.62	0.53	0.75	0.51	0.76	0.86	0.79	0.43	0.64	0.63	0.50	0.76	0.68	0.65	0.53	0.61	0.71	1.00								
19	0.47	0.36	0.45	0.36	0.44	0.47	0.46	0.30	0.40	0.44	0.35	0.49	0.45	0.46	0.43	0.45	0.38	0.46	1.00							
20	0.62	0.52	0.73	0.52	0.76	0.86	0.77	0.43	0.62	0.66	0.48	0.76	0.70	0.66	0.53	0.61	0.70	0.85	0.49	1.00						
21	0.42	0.44	0.46	0.47	0.49	0.48	0.46	0.35	0.46	0.45	0.41	0.45	0.46	0.51	0.38	0.41	0.44	0.45	0.33	0.47	1.00					
22	0.60	0.56	0.70	0.52	0.73	0.83	0.75	0.42	0.61	0.65	0.48	0.74	0.70	0.68	0.53	0.63	0.68	0.80	0.48	0.84	0.48	1.00				
23	0.59	0.55	0.73	0.49	0.73	0.82	0.75	0.42	0.62	0.61	0.52	0.72	0.65	0.68	0.52	0.60	0.71	0.81	0.46	0.81	0.45	0.77	1.00			
24	0.47	0.46	0.53	0.37	0.55	0.53	0.53	0.33	0.51	0.47	0.38	0.51	0.48	0.52	0.39	0.44	0.45	0.53	0.35	0.53	0.40	0.51	0.53	1.00		
25	0.49	0.47	0.59	0.46	0.59	0.64	0.61	0.41	0.55	0.55	0.41	0.58	0.52	0.52	0.44	0.50	0.68	0.63	0.40	0.61	0.42	0.61	0.63	0.43	1.00	
26	0.51	0.48	0.57	0.42	0.60	0.64	0.62	0.36	0.49	0.58	0.44	0.59	0.61	0.60	0.47	0.53	0.56	0.61	0.44	0.65	0.43	0.63	0.60	0.45	0.49	1.00

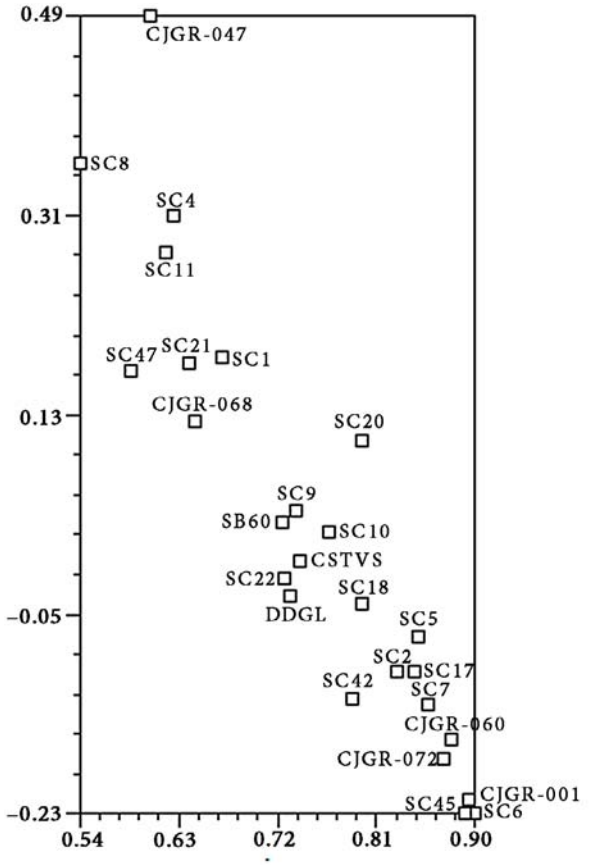

Figure 7. PCA plot estimates based on the Jaccard's similarity coefficients obtained from 369 AFLP fragments.

(2011) generated 809 polymorphic bands using 4 AFLP primer combinations and obtained an average of 202.3 polymorphic fragments per primer pair. This considerable difference with respect to our results can be explained by the previous authors' use of 2-base extensions to the specific selective primers used in their AFLP analysis. One to 2 selective nucleotides on the 3'-end of each selective primer may be suitable to show polymorphisms in small genomes (Blears et al., 1998) but may not be appropriate for members of *Crocus*, which have relatively large, poorly characterized genomes (Fernandez, 2004; Husaini et al., 2009). Brandizzi and Grilli Caiola (1997) reported that the estimated genomes of the triploid *C. sativus* and its 2 diploid most probable ancestors, *C. cartwrightianus* Herb. and *C. thomasii* Tenore, are approximately 3.47×10^9 bp. More complex genomes (i.e. those ranging from 10^8 to 10^9 bp) require the use of additional selective nucleotides in AFLP analysis to obtain the desired number of amplified fragments (Vos et al., 1995; Brandizzi and Grilli Caiola, 1997). Using other techniques, Grilli Caiola et al. (2004) found 2.1 polymorphic bands per primer pair and Beiki et al. (2010) acquired 3.8 polymorphic bands per primer pair when they compared diversity estimates obtained by RAPD in 7 *Crocus* species. Similarly, Moraga et al. (2010) obtained 4.8 polymorphic bands per primer in their ISSR study. Thus, AFLP primers tend to detect more polymorphisms per primer pair compared to RAPD and ISSR analyses.

Another objective of the present study was to identify the primer combinations that could have discriminatory power for the genetic identification of *Crocus* genotypes, as assessed by the PIC of each primer pair (Table 3). The highest PIC was obtained from primer combination M-CAT/E-AAG, suggesting that it could be highly useful for the study of genetic diversity between *Crocus* genotypes. Similarly, Erol et al. (2011) showed that AFLP primer pair M-CAT/E-AAG had the greatest discriminatory power in their study of *Crocus*.

The cluster analysis results obtained in the present study are broadly consistent with the findings of earlier diversity studies based on RAPD data (Beiki et al., 2010) and AFLP data (Zubor et al., 2004; Erol et al., 2011; Nazzal et al., 2011). However, our study included genotypes from regions that were not represented in the previous studies. A dendrogram generated from hierarchical UPGMA cluster analysis of Jaccard's similarity coefficient matrices revealed 4 major clades (Figure 6). Clades 1 and 4 contained only 1 genotype each, SC8 (*C. pallasii*) and SC60 (undefined taxon), respectively. All of the other *Crocus* genotypes were placed in clades 2 and 3. The Greek genotypes were found in clades 2 and 3 and did not exhibit common DNA fingerprints. Our dendrogram clearly indicated that *C. pallasii* (DDGL) shared the most AFLP fragments with *C. sativus*; this was in contrast to the dendrogram of Grilli Caiola et al. (2004), in which *C. sativus* was more related to *C. thomasii*. We did not observe a high correlation between genetic similarity and geographical distance.

The highest genetic similarity (0.86) was recorded between SC6 and CJGR-001, while the lowest similarity (0.30) was seen between SC8 and SC47. Alavi-Kia et al. (2008) reported that saffron and 3 wild *Crocus* species (*C. almehensis* Brickell & Mathew, *C. michelsonii* Fedtschenko, and *C. cancellatus* Herbert) showed a very high degree of similarity and could be distinguished by only 15 out of 28 IRAP markers. Zubor et al. (2004) used AFLP primers to classify 6 *Crocus* species and observed a close relationship between *C. sativus* and *C. cartwrightianus*. Moraga et al. (2010) observed no genetic variations among 12 ISSR markers and concluded that *C. cartwrightianus* cv. *albus* was more related to *C. sativus* than to *C. cartwrightianus*, and thus might be an albinic saffron. However, a RAPD-based study (Grilli Caiola et al., 2004) found high genetic diversity (genetic similarity = 0.33) between *C. pallasii* and *C. sativus*. Thus, our results are in agreement with those of Grilli Caiola et al. (2004). Furthermore, we calculated the average genetic similarity of *C. pallasii* genotypes that

Figure 8. Herbarium sheet of SC21: *Crocus pallasii* subsp. *dispathaceus*.

were collected from the same regions and found average genetic similarities of 0.48, 0.55, and 0.58 in the Aegean region of Turkey, the Mediterranean region of Turkey, and Greece, respectively. The highest level of genetic diversity (0.63) was reported for *C. pallasii* genotypes collected from the Central Anatolian region. Thus, the present results indicate the robustness of the AFLP technique in providing a higher degree of resolution for discriminating closely related genotypes within the species of *Crocus*.

A solid understanding of genetic variability and population structure is critical for the sustainable management and conservation of plant species (Sardaro et al., 2012). Several researchers have performed diversity and population structure analyses using various DNA marker techniques in different plants such as wheat (Chen et al., 2012), common bean (Asfaw et al., 2009), sesame (Cho et al., 2011), sunflower (Mandel et al., 2011), rice (Ming et al., 2010), and maize (Stich et al., 2010). The model-based structure analysis used herein revealed the presence of 2 populations among the collected genotypes. The grouping patterns obtained from the genetic similarity matrix and model-based membership differed somewhat (Figures 5 and 6). For example, some genotypes from cluster 1 of the structure analysis were placed in clades 2 and 3

of the NTSYS-based dendrogram. In the NTSYS-based dendrogram, which was based on the Jaccard similarity index, genotypes were not grouped by origin. These results were confirmed by Bayesian analyses, demonstrating that the *Crocus* genotypes have a complex genetic structure. The distribution of the 17 genotypes that shared at least >70% ancestry with 1 of the 2 inferred groups is summarized in Table 2. Another 34.7% of genotypes showed evidence of mixed ancestry, but the groupings were not consistent with the patterns of origin. Souza-Chies et al. (2012) used ISSR markers to examine the genetic structure within and among 4 complexes of *Sisyrinchium* L. species belonging to Iridaceae and observed a high amount of intrapopulation genetic variability and genetic structure. The genetic structure among germplasm collections consisting of hundreds of olive cultivars was recently characterized using SSRs (Koehmstedt et al., 2011) and AFLPs (Baldoni et al., 2006). However, the different methods of cluster analyses used in these studies (e.g., hierarchical clustering, PCA analysis, and structure analysis) failed to distinguish between olive cultivars of different origin. Similar population structure results (i.e. no clear geographic structure was detected using different clustering methods) were obtained using accessions of foxtail millet from different locations in an SSR study (Kim et al., 2012). In the present study, our AFLP-based PCA distributed the populations into 4 distinct groups (Figure 7) that were comparable with the branches produced by cluster analysis. Both the UPGMA dendrogram (Figure 6) and the PCA biplot (Figure 7) produced similar groupings among the 26 *Crocus* taxa.

Based on the results of nuclear DNA content analysis, the 2C DNA amounts of *Crocus* species investigated in this study varied from 5.08 pg (*C. asumaniae*) to 9.75 pg (*C. sativus*). These DNA values are similar to those obtained by Brandizzi and Grilli Caiola (1996, 1998). The small differences between the results of the 2 studies may be due to different protocols and internal standards. Our FCM analysis showed that the 2C nuclear DNA content of *C. pallasii* subsp. *pallasii* specimens varied from 5.45 pg (SC17) to 6.57 pg (DDGL) per nucleus, which is equal to about 1.18-fold intraspecific variation (Table 2). A similar type of nuclear DNA content variation was also observed in the perennial grass *Festuca pallens* Host. The species exhibits up to 1.17-fold variation for nuclear DNA content at both diploid and tetraploid levels (Smarda, 2006; Smarda and Bures, 2006). Bennetzen et al. (2005) suggested that the nature of the molecular processes was responsible for nuclear DNA content divergence among related taxa and the accumulation of relatively small changes due to retrotransposal activity. This indicates that nuclear DNA content diversification among species is a gradual process (Smarda et al., 2008). The 2C nuclear DNA contents of *C. pallasii* subsp. *turcicus* (SC22) and an undefined specimen (SB60) were 8.47 pg and 8.89 pg per nucleus, respectively (Table 2). These 2 specimens had much higher nuclear DNA content than *C. pallasii* subsp. *pallasii* specimens. Ohri (1998) suggested that nuclear DNA content differences within a genus can be useful in taxonomic classifications. Therefore, these 2 specimens may be treated as different species. Furthermore, our dendrogram based on AFLP analysis also showed that SB60 was genetically distinct from the other specimens.

Determining the levels of polymorphism and genetic diversity among *Crocus* genotypes from Turkey and Greece represents an important contribution towards conservation and the preservation of genetic diversity. AFLP markers are useful for assessing genetic diversity because they rapidly generate hundreds of highly replicable markers from the DNA of any organism, thereby allowing high-resolution, fingerprint-quality genotyping (Mueller and Wolfenbarger, 1999). In this study, we demonstrated the successful application of AFLP analysis to the study of genetic diversity and population structure among *Crocus* genotypes. Our data should be a valuable resource for breeding and genetic conservation programs in *Crocus*-growing regions.

This work revealed 6 important results:

1. *Crocus pallasii* subsp. *turcicus* cannot be distinguished by morphology but differs from its relatives by its 2C nuclear DNA content of 8.47 pg (2n = 14), as compared to the 5.73 pg per nucleus (±0.19) (2n = 16) of *Crocus pallasii* subsp. *pallasii*.

2. Specimen SB60 (8.89 pg) had a paler violet throat than *C. mathewii* and had different tepal shape, style, and color. Our analysis placed it in clade 4. After detailed morphological observation, we decided to publish SB60 as a new species in the near future.

3. The population of *C. pallasii* subsp. *dispathaceus* in Turkey seems to represent a new taxon or a new combination. The total 2C nuclear DNA of SC20 (Gülnar, Mersin Province) was 5.59 pg per nucleus while that of SC21 (Silifke, Mersin Province) was 6.75 pg, and they were placed into 2 different clades of the dendrogram. Thus, these genotypes appear to be distinct. In the future, we plan to study these specimens in more detail. The Syrian population of the taxon has distinctive brown tepals, whereas the Mersin, Turkey, population has purplish tepals.

4. The taxa of *Crocus asumaniae* and *C. mathewii* distributed in Turkey are also interesting. The specimens of *C. asumaniae* investigated in this study are morphologically and genetically variable (SC45 and SC47) (Figure 3). According to our observations, the original description fits only the specimens collected in the town of Cevizli near İbradı, Antalya. *C. mathewii* is a very rare taxon in

Turkey that can be found in only 2 different populations. The population near Kaş in Antalya Province (SC4) has been nearly eradicated by some foreign nurserymen. The population located in Muğla differs from the Antalya population by its morphology (tepals are larger and the flower shape is different). We aim to study both populations (SC4 and SC9) in detail in the near future.

5. The most interesting result of our study is that *Crocus sativus* and *C. pallasi* from a locality near Konya (SC42) are phylogenetically close to each other.

6. AFLP markers were highly efficient in detecting DNA polymorphisms over a large number of randomly sampled loci and were very useful in detecting high levels of genetic variation among *Crocus* genotypes. The identification of *Crocus* genotypes from Turkey and Greece contributes to our knowledge of genetic relationships and should facilitate strategies aimed at protecting natural populations and preserving genetic variability. Within the borders of Turkey, series *Crocus* shows an enormously diversified genetic structure. Additional investigations, such as morphometric measurements, cytological studies, and detailed field studies, will be needed to fully understand the taxonomy and biodiversity of this group in Turkey.

Acknowledgments

The first author is grateful to Dr Janis Ruksans for sharing his knowledge of the genus *Crocus* and for kindly sending specimens from Greece. We thank Prof Dr Orhan Küçüker (ISTF) for his scientific advice. We also thank MSc Almıla Çiftçi (ISTF) for preparing herbarium specimens. This work was supported by the Research Fund of İstanbul University, İstanbul, Turkey (project number: 27545).

References

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 Biotec Eq 22: 795–800.

Anderson JA, Churchill GA, Autrique JE, Tanksley SD, Sorrells ME (1993). Optimizing parental selection for genetic linkage maps. Genome 36: 181–186.

Arumuganathan K, Earle ED (1991). Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol Biol Rep 9: 229–241.

Asfaw A, Blair MW, Almekinders C (2009). Genetic diversity and population structure of common bean (*Phaseolus vulgaris* L.) landraces from the East African highlands. Theor Appl Genet 120: 1–12.

Baldoni L, Tosti N, Ricciolini C, Belaj A, Arcioni S, Pannelli S, Germana MS, Mulas M, Porceddu A (2006). Genetic structure of wild and cultivated olives in the central Mediterranean basin. Ann Bot-London 98: 935–942.

Beiki AH, Keifi F, Mozafari J (2010). Genetic differentiation of *Crucus* species by random amplified polymorphic DNA. Genetic Engineering and Biotechnology Journal 2010: 1–10.

Bennett MD, Leitch IJ (1995). Nuclear DNA amounts in angiosperms. Ann Bot-London 76: 113–176.

Bennetzen J, Ma J, Devos KM (2005). Mechanisms of recent genome size variation in flowering plants. Ann Bot-London 95: 127–132.

Blears MJ, De Grandis SA, Lee H, Trevors JT (1998). Amplified fragment length polymorphism (AFLP): a review of the procedure and its applications. J Ind Microbiol Biot 21: 99–114.

Botstein D, White RL, Skolnick M, Davis RW (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 32: 314–331.

Brandizzi F, Grilli Caiola MG (1996). Quantitative DNA analysis in different *Crocus* species (Iridaceae) by means of flow cytometry. Giornale Botanico Italiano 130: 643–645.

Brandizzi F, Grilli Caiola MG (1997). Calcium in the reproductive biology of *Crocus biflorus* Mill. subsp. *biflorus* (Iridaceae). Plant Biosyst 131: 69–74.

Brandizzi F, Grilli Caiola MG (1998). Flow cytometric analysis of nuclear DNA in *Crocus sativus* and allies (Iridaceae). Plant Syst Evol 211: 149–154.

Çelebioğlu S, Baytop ÖT (1949). Bitkisel Tozların Tetkiki İçin Yeni Bir Reaktif. İstanbul: Institute of Pharmacognosy (in Turkish).

Chen X, Min D, Yasir TA, Gang Hu Y (2012). Genetic diversity, population structure and linkage disequilibrium in elite Chinese winter wheat investigated with SSR markers. PLoS ONE 7: e44510.

Cho YI, Park JH, Lee CW, Ra WH, Chung JW, Lee JR, Ma KH, Lee SY, Lee KS, Lee MC et al. (2011). Evaluation of the genetic diversity and population structure of sesame (*Sesamum indicum* L.) using microsatellite markers. Genes Genom 33: 187–195.

Dolezel J, Binarova P, Lucretti S (1989). Analysis of nuclear DNA content in plant cells by flow cytometry. Biol Plantarum 31: 113–120.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12: 13–15.

Earl DA, von Holt BM (2012). STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4: 359–361.

Erol O, Küçüker O (2007). Leaf anatomy of some endemic *Crocus* L. (Iridaceae) taxa from the West Anatolia. Int J Bot 3: 290–295.

Erol O, Şık L, Kaya HB, Tanyolaç B, Küçüker O (2011). Genetic diversity of *Crocus antalyensis* B. Mathew (Iridaceae) and a new subspecies from southern Anatolia. Plant Syst Evol 294: 281–287.

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.

Fernandez JA (2004). Biology, biotechnology and biomedicine of saffron. Recent Research of Development in Plant Science 2: 127–159.

Grilli Caiola MG, Caputo P, Zanier R (2004). RAPD analysis in *Crocus sativus* L. accessions and related *Crocus* species. Biol Plantarum 48: 375–380.

Google Inc. (2012). Google Earth. Version 7.0.2.8415. Part of Anatolia, Thrace and East Aegean Islands. Available online at http://www.google.com/earth/index.html, visited on 02/04/2012.

Hamza H, Benabderrahim MA, Elbekkay M, Ferdaous G, Triki T, Ferchichi A (2012). Investigation of genetic variation in Tunisian date palm (*Phoenix dactylifera* L.) cultivars using ISSR marker systems and their relation with fruit characteristics. Turk J Biol 36: 449–458.

Harpke D, Meng S, Ruttun T, Kerndorff H, Blattner F (2013). Phylogeny of *Crocus* (Iridaceae) based on one chloroplast and two nuclear loci: ancient hybridization and chromosome number evolution. Mol Phylogenet Evol 66: 617–627.

Hubisz MJ, Falush D, Stephens M, Pritchard JK (2009). Inferring weak population structure with the assistance of sample group information. Mol Ecol Resour 9: 1322–1332.

Husaini AM, Wani SA, Sofi P, Rather AG, Parray GA, Shikari AB, Mir JI (2009). Bioinformatics of saffron (*Crocus sativus* L.) improvement. Communications in Biometry and Crop Science 4: 3–8.

Jaccard P (1908). Nouvelles recherches sur la distribution florale. Bulletin de la Société Vaudoise des Sciences Naturelles 44: 223–270.

Jonah PM, Bello LL, Lucky O, Midau A, Moruppa SM (2011). Review: the importance of molecular markers in plant breeding programmes. Global Journal of Science Frontier Research 11: 0975-5896.

Joshi SP, Prabhakar K, Ranjekar PK, Gupta VS (2011). Molecular markers in plant genome analysis. Current Science: 25 July. Available online at http/www.ias.ac.in/currsci/jul25/articles15.htm.

Kerndorff H, Pasche E (2011). Two new taxa of *Crocus* (Liliiflorae, Iridaceae) from Turkey. Stapfia 95: 2–5.

Kerndorff H, Pasche E, Harpke D, Blattner F (2011). Three new species of *Crocus* (Liliiflorae, Iridaceae) from Turkey. Stapfia 95: 19–25.

Kim EJ, Sa KJ, Cheul Park K, Lee JK (2012). Study of genetic diversity and relationships among accessions of foxtail millet [*Setaria italica* (L.) P. Beauv.] in Korea, China, and Pakistan using SSR markers. Genes Genom 34: 529–538.

Koehmstedt AM, Aradhya MK, Soleri D, Smith JL, Polito VS (2011). Molecular characterization of genetic diversity, structure, and differentiation in the olive (*Olea europaea* L.) germplasm collection of the United States Department of Agriculture. Genet Resour Crop Ev 58: 519–531.

Lee M (1995). DNA markers and plant breeding programs. Adv Agron 55: 265–344.

Mandel JR, Dechaine JM, Marek LF, Burke JM (2011). Genetic diversity and population structure in cultivated sunflower and a comparison to its wild progenitor, *Helianthus annus* L. Theor Appl Genet 123: 693–704.

Manel S, Schwartz MK, Luikart G, Taberlet P (2003). Land-scape genetics: combining landscape ecology and population genetics. Trends Ecol Evol 18: 189–197.

Mathew B (1982). The Crocus. A Revision of the Genus Crocus (Iridaceae). London: B. T. Batsford.

Mathew B (1984). *Crocus*. In: Davis PH, editor. Flora of Turkey and the East Aegean Islands, Vol. 8. Edinburgh: Edinburgh University Press, pp. 413–438.

Mathew B, Petersen G, Seberg O (2009). A reassessment of *Crocus* based on molecular analysis. New Plantsman 8: 50–57.

Meudt HM, Clarke AC (2007). Almost forgotten or latest practice? AFLP applications, analyses and advances. Trends Plant Sci 12: 106–117.

Ming H, Xie F, Chen L, Zhao X, Jojee L, Madonna D (2010). Comparative analysis of genetic diversity and structure in rice using ILP and SSR markers. Rice Science 17: 257–268.

Moraga AR, Trapero A, Ahrazem O, Gomez LG (2010). Crocins transport in *Crocus sativus*: the long road from a senescent stigma to a newborn corm. Photochemistry 71: 1506–1513.

Mueller UG, Wolfenbarger LL (1999). AFLP genotyping and fingerprinting. Trends Ecol Evol 14: 389–394.

Muthusamy S, Kanagarajan S, Ponnusamy S (2008). Efficiency of RAPD and ISSR markers system in accessing genetic variation of rice bean (*Vigna umbellata*) landraces. Electron J Biotechn 11: 1–10.

Nazzal KW, Shibli RA, Makhadmeh IM, Syouf MQ (2011). Amplified fragment length polymorphism (AFLP) analysis in *Crocus* spp. collected from Northern Jordan. Jordan Journal of Agricultural Sciences 7: 1–8.

Nemati Z, Zeinalabedina M, Mardi M, Pirseyediand SM, Marashi AH, Nekoui SMK (2012). Isolation and characterization of a first set of polymorphic microsatellite markers in saffron, *Crocus sativus.* Am J Bot 99: e340–e343.

Odong TL, van Heerwaarden J, Jansen J, van Hintum TJL, van Eeuwijk FA (2011). Determination of genetic structure of germplasm collections: are traditional hierarchical clustering methods appropriate for molecular marker data? Theor Appl Genet 123: 195–205.

Ohri D (1998). Genome size variation and plant systematic. Ann Bot-London 82: 75–83.

Petersen G, Seberg O, Thorsoe S, Jorgensen T, Mathew B (2008). A phylogeny of the genus *Crocus* (Iridaceae) based on sequence data from five plastid regions. Taxon 57: 487–499.

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, Rafalski A (1996). The comparison of RFLP, RAPD, AFLP, and SSR (microsatellite) markers for germplasm analysis. Mol Breeding 2: 225–238.

Poyraz İE, Sözen E, Ataşlar E, Poyraz İ (2012). Determination of genetic relationships among *Velezia* L. (Caryophyllaceae) species using RAPD markers. Turk J Biol 36: 293–302.

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

Rohlf FJ (2005). NTSYSpc (Numerical Taxonomy and Multivariate Analysis System), Version 2.2. New York: Exeter Software, Applied Biostatistics Inc.

Rubio-Moraga A, Trapero-Mozos A, Gómez-Gómez L, Ahrazem O (2010). Intersimple sequence repeats markers for molecular characterization of *Crocus cartwrightianus* cv. *albus*. Indus Crops Prod 32: 147–151.

Rudall P, Mathew B (1990). Leaf anatomy in *Crocus* (Iridaceae). Kew Bulletin 45: 535–544.

Sardaro MLS, Atallah M, Picarella ME, Aracri B, Pagnotta MA (2012). Genetic diversity, population structure and phylogenetic inference among Italian orchids of the *Serapias* genus assessed by AFLP molecular markers. Plant Syst Evol 298: 1701–1710.

Smarda P (2006). DNA ploidy levels and intraspecific DNA content variability in Romanian *fescues* measured in fresh and herbarium material. Folia Geobot 41: 417–432.

Smarda P, Bures P (2006). Intraspecific DNA content variability in *Festuca pallens* on different geographic scales and ploidy levels. Ann Bot-London 98: 665–678.

Smarda P, Bures P, Horova L, Foggi B, Rossi G (2008). Genome size and GC content evolution of *Festuca*: ancestral expansion and subsequent reduction. Ann Bot-London 101: 421–433.

Sönmezoğlu ÖA, Bozmaz B, Yıldırım A, Kandemir N, Aydın N (2012). Genetic characterization of Turkish bread wheat landraces based on microsatellite markers and morphological characters. Turk J Biol 36: 589–597.

Souza-Chies TT, dos Santos EK, Eggers L, Flores AM, Alves EMS, Fachinetto J, Lustosa J, Corrêa LB, Tacuatiá LO, Piccoli P et al. (2012). Studies on diversity and evolution of Iridaceae species in southern Brazil. Genet Mol Biol 35: 1027–1035.

Stich B, van Inghelandt D, Melchinger AE, Lebreton C (2010). Population structure and genetic diversity in a commercial maize breeding program assessed with SSR and SNP markers. Theor Appl Genet 120: 1289–1299.

Swanson T (1996). Global values of biological diversity: the public interest in the conservation of plant genetic resources for agriculture. Plant Genetic Resources Newsletter 105: 1–7.

Taşkın BG, Vardareli N, Doğaç E (2012). Genetic diversity of natural *Cyclamen alpinum* populations. Turk J Biol 36: 413–422.

Türktaş M, Aslay M, Kaya E, Ertuğrul F (2012). Molecular characterization of phylogenetic relationships in *Fritillaria* species inferred from chloroplast trnL-trnF sequences. Turk J Biol 36: 552–560.

Uzun FG, Kalender S, Durak D, Demir F, Kalender Y (2009). Malathion-induced testicular toxicity in male rats and the protective effect of vitamins C and E. Food Chem Toxicol 47: 1903–1908.

Vos P, Hogers R, Bleeker M, Reijans M, Lee T, Hornes M, Friters A, Pot J, Paleman J, Kuiper M et al. (1995). AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23: 4407–4414.

Winter P, Kahl G (1995). Molecular marker technologies for plant improvement. World J Microb Biot 11: 438–448.

Zhang L, Zhao H, Fan X, Wang M, Ding C, Yang R, Yin Z, Xie X, Zhou Y, Wan D (2012). Genetic diversity among *Salvia miltiorrhiza* Bunge and related species inferred from nrDNA ITS sequences. Turk J Biol 36: 319–326.

Zubor AA, Suranyi G, Gyori Z, Borbely G, Prokish J (2004). Molecular biological approach of the systematics of *Crocus sativus* L. and its allies. Acta Hortic 650: 85–93.