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A STUDY OF GENETIC RELATIONSHIPS OF *CROCUS* TAXA, SERIES *BIFLORI* (IRIDACEAE) FROM BULGARIA BY ISSR MARKERS

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Abstract

The taxonomy of the genus *Crocus* ser. *Nudiscapus* and the relationship between species in the genus are complicated and often contradictory. In this study, the genetic diversity and molecular markers' pattern variation among members of the *C. biflorus* group in Bulgaria were evaluated. For this study, five *Crocus* L. species from 15 natural populations were collected and assessed using the ISSR marker system. The data obtained was consolidated in a consensus tree, revealing a high degree of genetic variability among the studied species, as well as among the specimens inside the group.

Key words: ISSR, *Crocus*, *C. biflorus*, sect. *Nudiscapus*

Introduction. *Crocus biflorus* s.l. belonging to the *C.* sect. *Nudiscapus* B. Mathew, ser. *Biflori* B. Mathew is one of the most heterogenic and challenging group in the genus *Crocus*. *Crocus biflorus* consists of several taxa ranked as subspecies or varieties by different authors. The morphological classification is often insufficient to identify hidden taxa and solve deep taxonomical relationships among them, especially when inhabited areas are large and heterogeneous like

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in the Balkans, including Bulgaria. Therefore, a number of discrepancies in the classifications based on morphological characteristics of the species have been reported.

According to recent floristic data, the genus *Crocus* is represented in Bulgaria by nine well-defined, distinct species [1]. The taxonomy of the genus in Bulgaria [2] has not been revised or updated since 1964. All Bulgarian members of the sect. *Nudiscapus* are classified as *C. biflorus* – plants with white or pale-bluish to pale-lilac flowers and 3–4 longitudinal dark stripes on the outer perigon segments [3].

In the earlier editions of Flora of Bulgaria [2], *C. biflorus* is represented only by one variety: *C. biflorus* var. *violaceus* Boiss, while in the last version of Flora of Bulgaria [4], *C. biflorus* is represented by three different varieties: var. *biflorus*, var. *adamii* (Gay.) Bak. and var. *albus* Herb. Very often, *C. biflorus* subsp. *adamii* and *C. biflorus* var. *violaceus* are used interchangeably.

The group of *C. biflorus* s.l. is widely distributed in the Balkans – Greece, Rhodos, Turkey [5], and Bulgaria [2]: two-coloured early spring crocuses, with leathery-membranous bulbous shell scales and detachable ring. However, despite the intensive studies, the classification of this group remains unclear. So far, Bulgarian *Crocus* populations have not been a subject of intensive molecular taxonomy studies.

Here we describe our attempt to clarify the taxonomy of the *C. biflorus* s.l. group in Bulgaria using morphological and ISSR profiling of 15 different Bulgarian populations.

Materials and methods. Samples from fifteen Bulgarian populations of sect. *Nudiscapus* were collected between 2019 and 2020. The identification of the taxa was made according to the protologues, determination keys, and recent referent sources cited above. *C. adamioides* and *C. ranjeloviciorum* were included in the study as the most closely related species of ser. *Biflori*. *Crocus flavus* was used to define the outgroup in the Neighbour joining analysis. The voucher specimens were deposited in the herbarium of the Agricultural University – Plovdiv (SOA). Ex-situ specimens used in the study were kindly provided by the Institute of Plant Genetic Resources, Sadovo, Bulgaria.

Voucher specimens. Description of the voucher specimens used in the study is provided in Table 1. Above-ground parts from 10 randomly collected individuals from all analyzed populations were stored at $-80\,^{\circ}\mathrm{C}$ for subsequent molecular analysis.

Genomic DNA extraction. Plant genomic DNA was extracted and purified using DNeasy Plant Mini Kit (QIAGEN, Germany). Briefly, 100 mg of plant material was ground in liquid nitrogen and processed according to the manufacturer's requirements. Total DNA was quantified by UV spectrophotometer (Epoch™ Microplate Spectrophotometer, USA), and DNA integrity was evaluated on 1% agarose gel electrophoresis.

Table 1

Voucher specimens used in the study

Species	Location and GPS coordinates	Voucher ID
Crocus adamioides Kernd. & Pasche	Thracian lowland: 35TLG86. Near "Propadnaloto-Blato" protected locality, N42.12545 E25.64678, 145 m	SOA 062524
Crocus agg. *Biflorus*	Black Sea coast (South): 35TNG68. Cape Maslen Nos, peak Kitka, N42.30571 E27.74928, 130 m	SOA 062712
Crocus agg. *Biflorus*	Forebalkan (East): 35TLH87. Tarnovo highlands, Preobrazhenski Monastery, N43.120309 E25.606662, 340 m	SOA 062816
Crocus agg. *Biflorus*	Balkan Range (Central): 35TLH75. Via Ferrata trail near the monastery of Dryanovo, N42.94689 E25.435055, 345 m	SOA 062817
Crocus agg. *Biflorus*	Balkan Range (East): 35TMH42. Meten Kamak locality near Sliven, N42.70384 E26.33687, 423 m	SOA 062725
Crocus agg. *Biflorus*	Balkan Range (East): 35TNH22. Genger locality, near Aytos, N42.71256 E27.26865, 169 m	SOA 062794
Crocus agg. *Biflorus*	Tundzha Hilly Plain: 35TMG43. Scrubs near the village of Moustrak, N41.87795 E26.29031, 306 m	SOA 062715
Crocus chrysanthus Herb.	Rhodopi Mts (Central): 35TKG95. Oak forest between the town of Perushtitsa and the village of Skobelevo, N42.02534 E24.54629, 812 m	SOA 062598;
Crocus chrysanthus Herb.	Rhodopi Mts (Central): 35TLG24. Anathema locality, near the town of Asenovgrad, N41.97386 E24.91066, 719 m	SOA 062597
Crocus chrysanthus Herb.	Rhodopi Mts (East): 35TMF08. Near the village of Kazak, N41.4099167 E25.8826944, 188 m	SOA 062603
Crocus chrysanthus Herb	Thracian Lowland: 35TKG96. Bessapara ridges, an open slope above the village of Novo Selo, N42.10026 E24.47349, 262 m	SOA 062600
Crocus chrysanthus Herb.	Tundzha Hilly Plain: 35TMG66: Oak forest near the village of Chernozem, N42.10271 E26.59859, 193 m	SOA 062843.
Crocus pallidus Kitan. & Drenk.	Black Sea coast (North): 35TPJ21. Oak grove near the village of Kamen Bryag, N43.45199 E28.54402, 41 m	SOA 062791
Crocus randjelovicio-rum Kernd., Pasche, Harpke & Raca	Balkan Range (West): 34TFN76. Petrohan Narrow, under Shilny Peak, N43.01154 E23.12118, 1005 m	SOA 062856
Crocus flavus West.	Tundzha Hilly Plain: 35TNG57. Oak forest, locality of Anatemska-Dolina, near the village of Yasna Polyana, N42.26274 E27.70973, 15 m	SOA 062708

Table 2

List of primer sequences used for ISSR analysis in this study

No	Primer name	Sequence 5' → 3'	Total numbers	Mono-morphic bands	Poly-morphic bands
1.	L1	CACACACACACACACAA(R)G	15	5	10
2.	L2	GAGAGAGAGAGAGAGA(Y)C	7	3	4
3.	L3	AGAGAGAGAGAGAGAG(Y)C	8	2	6
4.	L6	AGAGAGAGAGAGAGAGC	10	2	8
5.	L7	CTCTCTCTCTCTCTC(K)C	10	4	6
6.	L8	AGAGAGAGAGAGAGAG(V)C	10	7	3
Total		60		23	37
Average		10		3.83	6.17
% Polymorphisms		61.67			

Note: R = A + G; Y = C + T; V = G + A + C; K = T + G

ISSR analyses. The six ISSR primers selected were used to amplify the genomic DNA extracted from fifteen Bulgarian populations of sect. *Nudiscapus* (Table 2). The amplification of the ISSR loci was carried out in a 25 µl PCR reaction mixture consisting of 2.5 µl 10 × PCR buffer, 30 ng template DNA, 3.0 mM $MgCl_2$, 0.1 mM dNTP, 10 pmol primers, and 1.0U DreamTaq DNA Polymerase (ThermoFisher, USA). PCR reactions were performed in a T100 Thermal Cycler (Bio-Rad, USA) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 40 cycles of 20 s at 94 °C, 60 s at 55 °C, and 80 s at 72 °C, followed by final product extension for 6 min at 72 °C. The negative control was run by replacing template DNA with ddH_2O to test for the possibility of contamination. The amplification products were separated in 2.0% agarose gels stained with ethidium bromide and photographed with Gel (BioRad, USA). Molecular weights were estimated using a 100 bp Plus DNA ladder (ThermoFisher, USA).

The data from the ISSR marker analysis was scored for presence (1) and absence (0) of bands. Weak and unclear bands were not counted. The PAST 4.05 software was used to analyze the genetic diversity among the studied species, using clustering by the Neighbour joining method with a defined outgroup [6]. The consensus dendrogram was constructed using the CONSENSE application, part of the PHYLIP 3.69 software [7].

Results. Six ISSR primers were used to produce DNA fingerprint profiles (Table 1). Out of the amplified 60 loci, 37 were polymorphic, reflecting rich allelic diversity among *Crocus* species. The size of the amplified bands ranged between 200 bp and 900 bp. A total of 60 markers were analyzed (Table 2). The number of scored bands for each primer varied from 7 (L2) to 15 (L1), with a mean number of 6 markers per primer. This multiplex ratio (total of detected bands/total

number of primer combinations) value (6) was similar to values obtained with ISSRs for other species such as *Trigonella* spp. with a value of 7.3 [8]. Thirty-seven polymorphic bands were observed and ranged from 3 (L8) to 10 (L1) per primer. Each primer generated, on average, 6.17 polymorphisms.

The obtained results from the genetic polymorphism analysis were used to construct individual trees using the Neighbour joining method (Fig. 1). The best clustering of the species was most clearly seen in the tree's topology, obtained from the amplification using primer sequences L1, which amplifies the highest number of alleles (Table 2). A similar clade structure was obtained using primers L2 and L3. Therefore, the trees obtained from the L6 and L8 group, the specimens by intraspecific differences, were not limited by the taxonomy.

As shown in Fig. 1, in four phylogenetic trees, built based on data obtained from primers L1, L2, L3, and L7, the samples of *C.* agg. *biflorus* are clustered together in a separated clade. However, on the trees built using data from L1 and L2 primers, species determined as *C. adamioides* (0625243), *C. randjeloviciorum* (062856), and a *C. biflorus* (062816) are located far from their putative taxonomically close species. High genetic variability of *C. chrysanthus* was observed in all six trees (Fig. 1). In the case of L1, this species does form its own branch. The

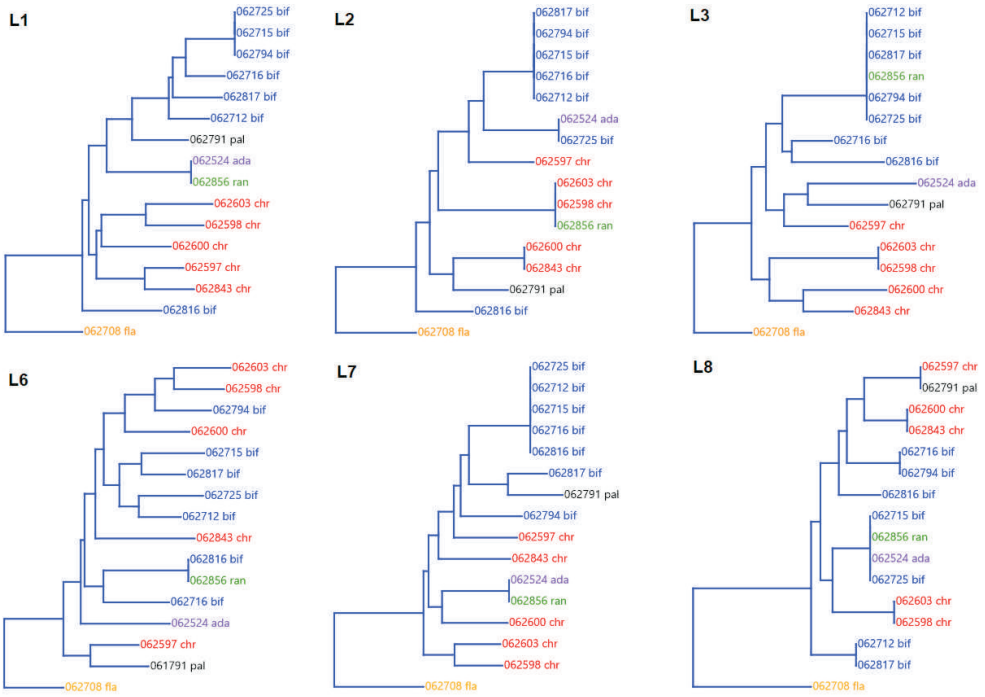

Fig. 1. Dendrograms of different *Crocus* samples of ser. *Nudiscapus*, Neighbour joining clustering method for each used ISSR primer (L1–L8)

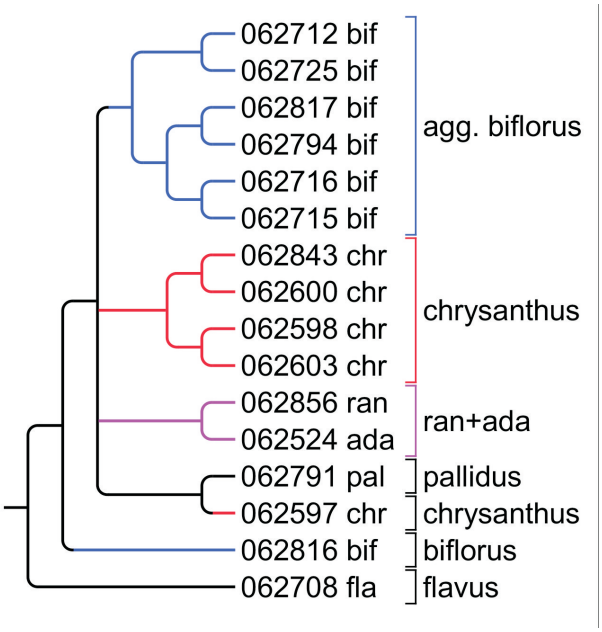

Fig. 2. Dendrogram of different *Crocus* samples using Unrooted consensus tree built CONSENSE (PHYLIP)

high degree of genetic variability in all other primers sets resulted in clustering in two well-distinguished clades. On the tree, representing the ISSR pattern obtained using two of the less conservative primers (L6 and L8), *C. pallidus* (062719) is clustered together with *C. chrysanthus* (062597).

In the final consensus tree (Fig. 2), all samples were determined as *C.* agg. *biflorus* and C. *chrysanthus* are clustered into separate well-defined clades. In the consensus dendrogram, *C. randjeloviciorum* and *C. adamioides* form a distinct clade. A similar different branch is created by *C. pallidus* and *C. chrysanthus.* One of the specimens included in the analysis, determined as *C. biflorus* (062816), shows a significant genetic distance from other species and does not cluster in any clade. The second one consisted of a group of taxa with morphologically similar characters. The analysis displayed a higher similarity between *C. adamioides* and *C. randjeloviciorum* (L1, L7, L8) than the other evaluated members.

Discussion. The consensus dendrogram obtained by cluster analysis of the data obtained by the ISSR marker system shows a clear grouping of samples belonging to *C. biflorus* and *C. chrysanthus.* Although determined as *C. chrysanthus* and *C. biflorus,* one sample of each species does not cluster in an appropriate clade, which can be due to a high degree of divergence of populations. Our finding agrees with previous phylogenetic studies of these taxa based on chloroplast and two nuclear loci [9].

In our hands, the grouping of the evaluated populations of *C. ser. Nudiscapus*, based on single ISSR markers, displayed a high level of genetic variation, especially primers L6 and L8. The high degree of genetic molecular dispersion observed in the *C.* c.f. *biflorus* samples corresponds to the morphological polymorphism within the group. As expected, significant genetic differences were found between *C. pallidus* and *C. chrysanthus*. Most consistent clusterings were achieved with primers L1 $(CA)_8A(R)G$, L2 $(GA)_8(Y)C$, and L3 $(AG)_8(Y)C$.

Conclusion. Although the relationships between the different clades are in some cases weak to moderate, they support their independence and taxonomical status. Our results indicated that the taxonomic structure of *C. biflorus* s.l. accepted until recently needs a significant revision. In this respect, the ISSR marker system can provide a valuable and easily accessible tool for genetic analysis of *Crocus* species and solving taxonomical ambiguities.

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