GENETIC DIFFERENTIATION BETWEEN SPECIES OF THE GENUS CROCUS BASED ON THE ISOENZYME ANALYSIS

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Abstract

This preliminary study represents data for the electrophoretic variations in the expression of the enzyme superoxide dismutase in six species of genus Crocus. Polymorphism at two superoxide dismutase loci was reported, with two alleles at the locus Sod-1 (Sod-1^{100} u Sod-1^{89}) and three alleles at the locus Sod-4 (Sod-4^{100}, Sod-4^{90} u Sod-4^{78}). A comparative analysis of allelic frequencies was made and the degree of genetic differentiation between the studied species was characterized. The genetic distance according to Nei was determined and the phylogenetic dependences at the interspecific level were analyzed.

Keywords: *isozymes, superoxide dismutase, genus Crocus, genetic distance*

1. INTRODUCTION

Genetically determined biochemical polymorphism of enzymes has been used successfully to study genetic heterogeneity in populations, as well as for interspecific differentiation in plants (Crawford 1983; Angelov 2003).

The taxonomy of the genus *Crocus* in Bulgaria needs updating. There are no up-to-date data on the structure of Bulgarian species, as well as the variability within polymorphic species (Raycheva et al. 2021).

Most plant species contain numerous isoforms of superoxide dismutases, which differ in their active metal ions. Each of these isoforms is encoded by different genes belonging to the same family, whose transcriptional regulation is controlled by intracellular and extracellular factors.

The identification of crocuses based on their genotypic differences by molecular markers such as isoenzymes is of great importance for understanding the biological diversity in this genus. In the present work, isoenzyme variations of superoxide dismutases are used as a genetic marker to identify the studied species of the genus *Crocus* L. (*Iridaceae*), as well as to determine the genetic similarity and distance between them.

Superoxide dismutases (SOD) (EC 1.15.1.1) catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide, which are released into the medium or used as a substrate for other enzymes. They play a major role in protection against toxic oxygen radicals, which are generated as by-products of many metabolic oxidative processes. Several studies have shown that the high activity of SOD and the greater number of isoforms determine the greater potential for the removal of toxic radicals (Berwal & Ram, 2019). Several studies of superoxide dismutase isoenzymes in plants have been performed and interspecific and interspecific differences have been reported (Reddy & Venkaiah B., 1982; Hernandez et al., 2006; Berwal et al., 2016). Heterozygosity, considered an indicator of genetic diversity, is a convenient parameter for studying genetic variability (Ting Ji & Cohong, 2011).

Based on the metal co-factor used by the enzyme, superoxide dismutases are classified into three groups: iron-containing (Fe-SOD), manganese-containing (Mn-SOD), and copper and zinc-containing (Cu / Zn-SOD). They are localized in different parts of the cell: Fe-SOD is found in chloroplasts, Mn-SOD in mitochondria and peroxisomes, and Cu / Zn-SOD in chloroplasts, cytosol, and extracellular space (Alscher et al., 2002).

In the present study, isoenzyme variations of superoxide dismutases are used as a genetic marker to identify the studied species of the genus *Crocus*, as well as to determine the genetic similarity and distance between them.

2. MATERIALS AND METHODS

2.1. Materials

According to the objectives of the planned study, 443 individuals were used from Crocus pallasii Goldbl., C. chrysantus Herb., C. adamioides Kernd. & Pasche, C. pulchellus Herb., C. flavus West. and C. cf. biflorus auct. bulg. The plants were collected from 33 natural localities in Bulgaria. Voucher specimens were deposited in the Herbarium of Agricultural university – Plovdiv (SOA):

C. chrysanthusHerb.:

Rhodopi Mts. (central): **35TKG95.** Rocks above the town of Peroushtitsa, 661 m, N42.027826 E24.532109, 2020-01-25 (coll. T.Raycheva), SOA s/n [2020.210 (2020.010)]; 808 m, N42.030938 E24.534379, 2020-02-22 (coll. T.Raycheva & K. Stoyanov), SOA 062739 [2020.222]

Thrakian lowland: **35TKG96.** Bessapara Hills, peak Shirokiya Vruh, 262 m, N42.10026 E24.47349, 2019-02-17 (coll. Raycheva & Stoyanov), SOA 062600 [2019.021]

C. pulchellus Herb.:

Rhodopi Mts. (east): **35TLF79**. the village of Karamfil, N41.4767 E25.5483 521 m, 2019-11-15, (coll. Ts. Raycheva & K. Stoyanov), SOA 062674 [2019.104].

Thrakian lowland**:** **35TLF79**. Karamfil, 521 m, N41.4767 E25.5483, 2019-11-15, (coll. Raycheva & Stoyanov), SOA 062674 [2019.104]; 35TLG85. Brod, 127 m, N42.05697 E25.66899, 2019-10-09 (coll. Ts. Raycheva & K. Stoyanov) SOA s/n [2019.096]. **35TLG86**. Near "Propadnaloto Blato" protected locality, Quercus forest. 118 m, N42.12439 E25.6417, 2019-10-09 (coll. T.Raycheva & K.Stoyanov), SOA s/n [2019.095]

C. pallasii Goldb.:

Trakian lowland: **35TKG86**. Bessapara Hills, Elenski Peak, 290 m, N42.13784 E24.37206, 2018-11-14 (coll. K. Stoyanov), SOA 062443 [2018.004a]; 408 m, N42.12516 E24.40894, 2018-11-14 (coll. K. Stoyanov), SOA 062444 [2018.004b]; 320 m, N42.12598 E24.4345, 2018-11-14 (coll. K. Stoyanov), SOA 062445 [2018.004c]; **35TKG96**. Bessapara Hills, the village of Novo Selo, on the northern slope of the peak Shirokiya Vruh, in shrubs. 2019-11-10 (coll. T.Raycheva & K.Stoyanov), SOA s/n [2019.101]; **35TLG16**. Hill of Youth, Plovdiv, 199 m, N42.13596 E24.72913 (coll. K. Stoyanov), SOA 062432 [2018.002].

C. flavus West.:

Black Sea Coast (southern): **35TLF79**. the village of Sokolino, 400 m, N41.52492 E25.44383, 2020-03-05, (coll. V. Trifonov), SOA 062821 [2020.132]. **35TLG83**. Gulubetsh village, 350 m, N41.8275 E25.635, 2020-02-29 (coll. V. Trifonov) SOA 062815 [2020.118]; **35TNG39**. Mandra Lake, the Chekiliyata heights (northern), in a forest of Quercus frainetto,8 m, N42.40274 E27.44580, 2020-02-15 (coll. T.Raycheva & K.Stoyanov), SOA 062801 [2020.033].

C. adamioides Kernd. & Pasche:

Balkan Range (eastern): **35TMH42**. The locality Meten Kamak, Sliven, 423 m, N42.70384 E26.33687, 2020-01-17 (coll. I. Kostadinov, Ts. Raycjheva, K. Stoyanov) SOA 062725 [2020.001].

Toundja Hilly Plain: **35TMG56**. The locality "Golemiya Kamak" near the village of Knyazhevo, 130 m, N42.09814 E26.50484, 2020-02-03 (coll. Ts. Raycheva & K.Stoyanov), SOA 062719 [2020.015*]*

C. cf. biflorus:

Toundja Hilly Plain: **35TMH22.** Tepyata, the protected locality "The island of Toundja river", 256 m, N42.63839 E26.13462, 2020-01-17 (coll. I. Kostadinov, Ts. Raycheva, K. Stoyanov), SOA 062718 [2020.005], **35TMH31**. the village of Kovachite, 270 m, N42.56028 E26.19053, 2020-02-10 (coll. I. Kostadinov), SOA 062736 [2020.026]; 35TMH40. Chokoba, 255 m, N42.51742 E26.33233, 2020-02-16 (coll. I. Kostadinov), SOA 062836 [2020.044]; 35TMH41. Panaretovtsi, 240 m, N42.55333 E26.29883, 2020-02-16 (coll. I. Kostadinov), SOA 062837 [2020.045]; **35TMH42**. Barmouk hill, 345 m, N42.68587 E26.30112, 2020-02-17 (coll. Ivan Kostadinov), SOA 062727 [2020.042], Meten Kamak locality, 423 m, N42.70384 E26.33687, 2020-01-17 (coll. I. Kostadinov, Ts Raycheva & K. Stoyanov) SOA 062725 [2020.001].

2.2. Methods

The samples are examined by electrophoresis in polyacrylamide gel by the method of Maurer (1971), with modifications according to Ivanova (1996). The development of superoxide dismutases was performed according to Korochkin's method 1977). A double-layer gel was used - concentrating and separating. Based on the performed experiments, the variant of 11% concentration of the separating polyacrylamide gel was accepted as a model. The flowers were smeared in Eppendorf tubes in tris-phosphate buffer and left to extract for 18 hours at 4°C. The homogenate was centrifuged for 15 minutes at 5000 rpm. For the electrophoretic analysis, 10 µl of each sample was added dropwise. The development of superoxide dismutases was carried out following the protocol of Korochkin et al. (1977). When stained on a dark background, white bands appear, reflecting the localization of the enzyme.

The allelic frequency, polymorphism, levels, and genetic distance by Nei (1972) were calculated using the BIOSYS-1 software package (Swofford & Selander, 1981), through which are calculated: allelic frequencies, the average number of locus alleles, the degree of polymorphism, established and expected heterozygosity. The UPGMA phylogram was built using PHYLIP v.3.68 software package (Felsennstein, 1993), and visualized using TreeGraph2.

3. RESULTS AND DISCUSSION

Our electrophoretic analysis showed variability of the SOD system in the investigated species. The pronounced multiple forms of SOD are divided into four zones (SOD-1, SOD-2, SOD-3, and SOD-4), following their decreasing electrophoretic mobility. The expression and combination of the fractions from these zones determine the likely genetic control of superoxide dismutases from four loci.

In the first zone of the spectrum of the studied individuals of the species *C. pulchellus*, two fractions with high electrophoretic mobility were visualized. The nature of the expression of the two fractions gives us reason to comment on the action of a polymorphic locus Sod-1, represented by two alleles (Sod-1100 and Sod-189). In individuals from the other studied species of the genus Crocus, we reported monomorphism at this locus.

In the second and third zones of the spectrum of the individuals of the studied species one fraction is visualized (Fig. 1-6). The lack of variability in the expression of superoxide dismutases in these areas determines the probable monogenic control of monomorphic loci – Sod-2 and Sod-3. The nature of the expression of multiple forms of SOD in the fourth zone of the spectrum gives us reason to assume gene control from a polymorphic locus Sod-4, with the presence of 3 codominant alleles – Sod-4100, Sod-490, and Sod-478. All three alleles were present in the gene pool of *C. flavus*, as in the genotypes of the studied individuals their homozygous combinations prevailed. We also reported heterozygotes of the type Sod -4100/490, Sod -4100/478, and Sod -490/478 (Fig. 3).

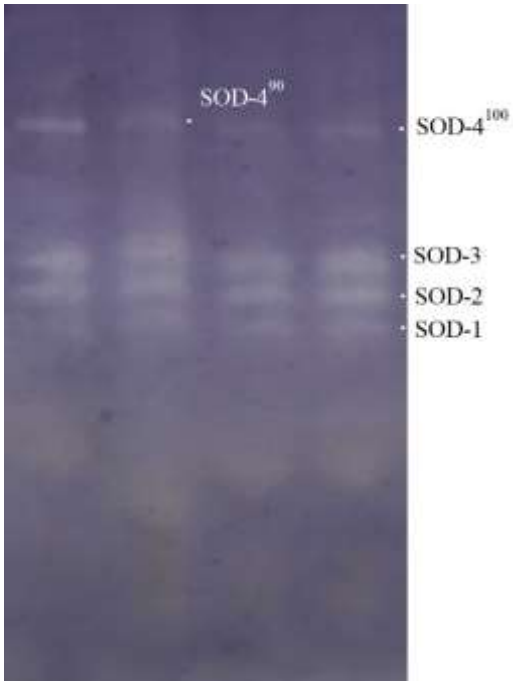

Fig. 1. Spectrum of SOD in *Crocus chrysantus.*

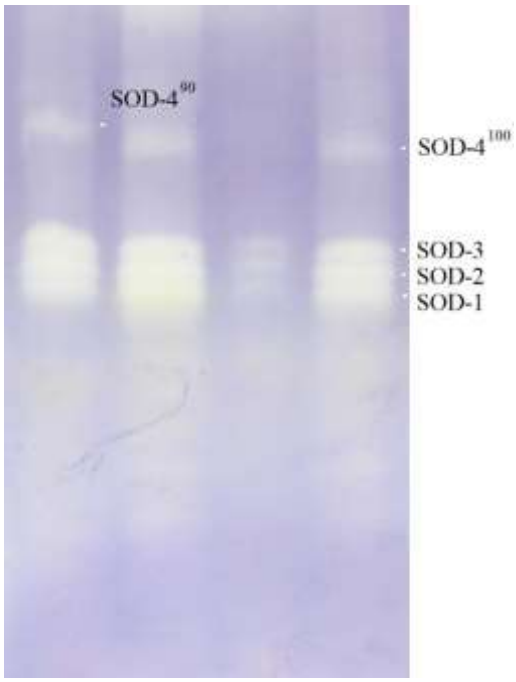

Fig. 2. Spectrum of SOD in *Crocus pallasii.*

In the other studied species of the genus *Crocus*, we found a dual allelic polymorphism at the locus Sod-4, represented by alleles Sod-4100 и Sod-490.

In the genotypic structure of the *C. biflorus*, we found only homozygotes on the two different alleles. A high percentage of heterozygotes of the type Sod-4100/490 was reported in *C. pulchellus* and *C. pallasii*.

Due to the dimeric structure of the enzyme, in some electrophoregrams, we observed the appearance of intermediate fractions, which are interlocus heterodimeric products.

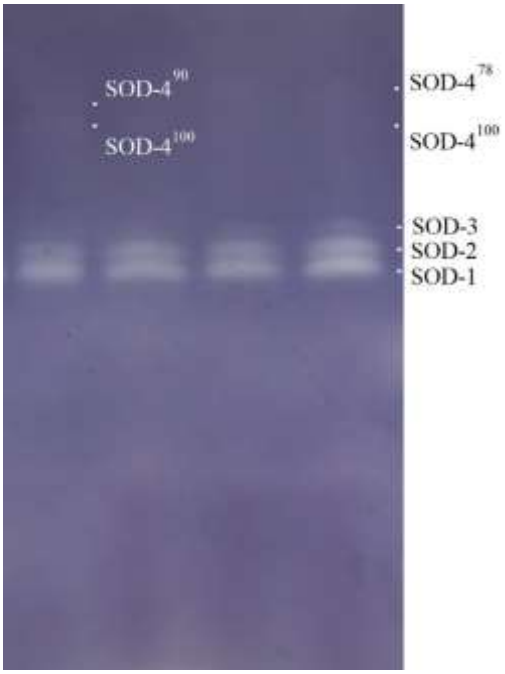

Fig. 3. Spectrum of SOD in *Crocus flavus.*

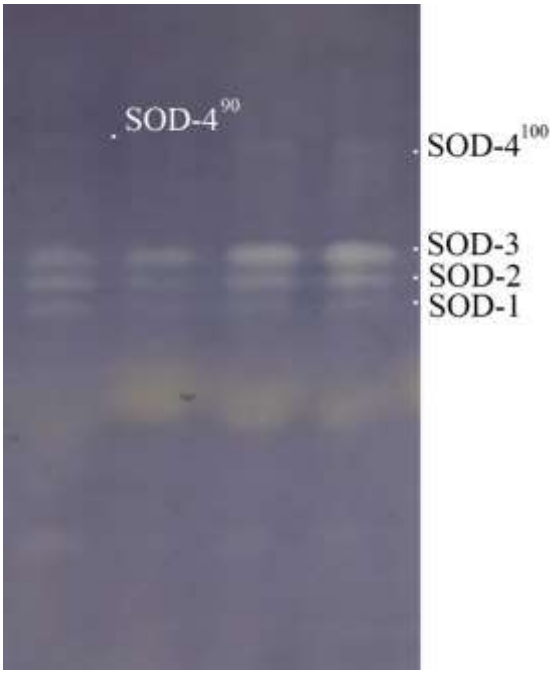

Fig. 4. Spectrum of SOD in *Crocus biflorus.*

We found species specificity of the genetic control of superoxide dismutases in *C. pulchellus*.

The model of isoforms in this species showed the expression of products of four Sod loci, but some of them have different electrophoretic mobility compared to the other studied species of the genus Crocus. For example, in the spectrum of individuals of the species *Crocus pulchellus* the product of locus Sod-2 is missing, but a new fraction with lower electrophoretic mobility was shown, showing expression of another locus – Sod-4pul (Fig. 5). This result determines the superoxide dismutases as a useful biochemical-genetic marker for the characterization and discrimination of *C. pulchellus*.

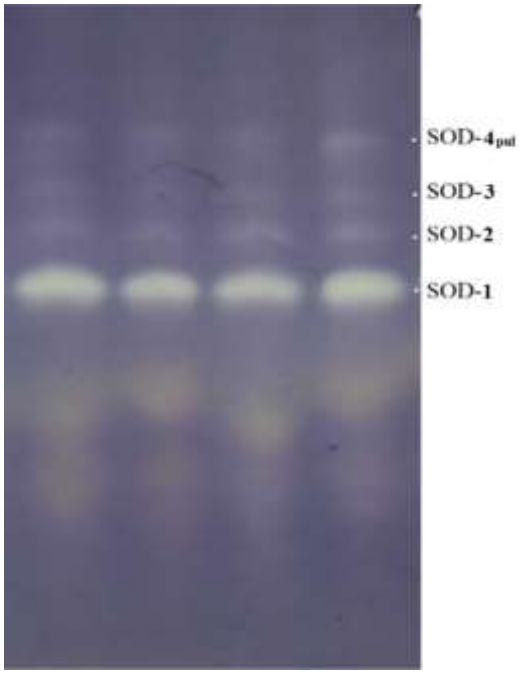

Fig. 5. Spectrum of SOD in *Crocus pulchellus.*

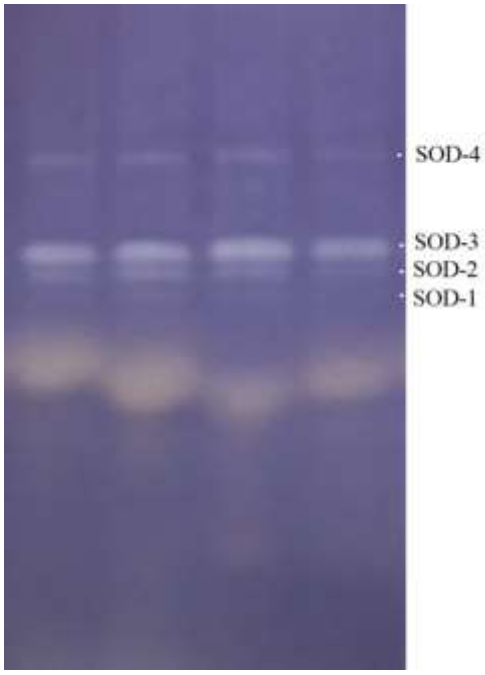

Fig. 6. Spectrum of SOD in *Crocus adamioides.*

We detected also differences in the intensity of the fractions in the SOD zones for the different species of crocuses. The variation in the expression of the Sod-3 locus is visible. High intensity of the product of this locus was found in the species *C. adamioides* and *C. biflorus* (Figs. 4 and 6), while in the species *C. flavus* the same fraction showed a very weak expression (Fig. 3). The highest expression of the Sod-1 locus was observed in individuals of the species *Crocus pulchellus* (Fig. 5), in the electrophoregrams of which the corresponding fraction was very intense. In the spectrum of individuals of the species *C. pallasii*, SOD isoenzymes showed similar intensity.

We assume that the observed differences in the intensity of the fractions in the SOD zones for the individual species are determined by the different ecological conditions. This finding is consistent with that found by Johnson et al. (1969) dependence of the variability of enzyme systems on environmental conditions.

Crocus pulchellus is the only representative of the genus with white anthers in Bulgaria. This morphological feature is related to the specificity in the genetic control of SOD.

The degree of polymorphism was highest in *C. pulchellus* (0.50). The mean number of locus alleles ranged from 1.3 to 1.5 (Table 1). The expected heterozygosity was higher than that obtained in all studied species of the genus. The highest level of observed heterozygosity was observed in *C. pulchellus* (0.120).

Species	**Mean sample size**	**Mean number of alleles for locus**	**Degree of polymorphism**	**Established heterozygosity**	**Expected heterozygosity**
Crocus pallasii	84	1.3 ±0.3	25.0	0.030	0.124
Crocus chrysantus	47	1.3 ±0.3	25.0	0.016	0.115
Crocus pulchellus	50	1.5 ±0.3	50.0	0.120	0.155
Crocus adamioides	52	1.3 ±0.3	25.0	0.014	0.126
Crocus flavus	108	1.5 ±0.5	25.0	0.056	0.139
Crocus* cf. *biflorus	102	1.3 ±0.3	25.0	0.000	0.112

Table 1. Average sample size and the average number of locus alleles, degree of polymorphism, established and expected heterozygosity.

Determination of genetic distance is used for phylogenetic comparison by constructing schemes based on the results of isoenzyme analysis. The built phylogram (Fig.7), represented the relationships between the investigated species of genus *Crocus* and divided *C. pulchellus* far from the other species. The same division could be observed morphologically because *C. pulchellus* has white anthers, and all other investigated species have yellow anthers.

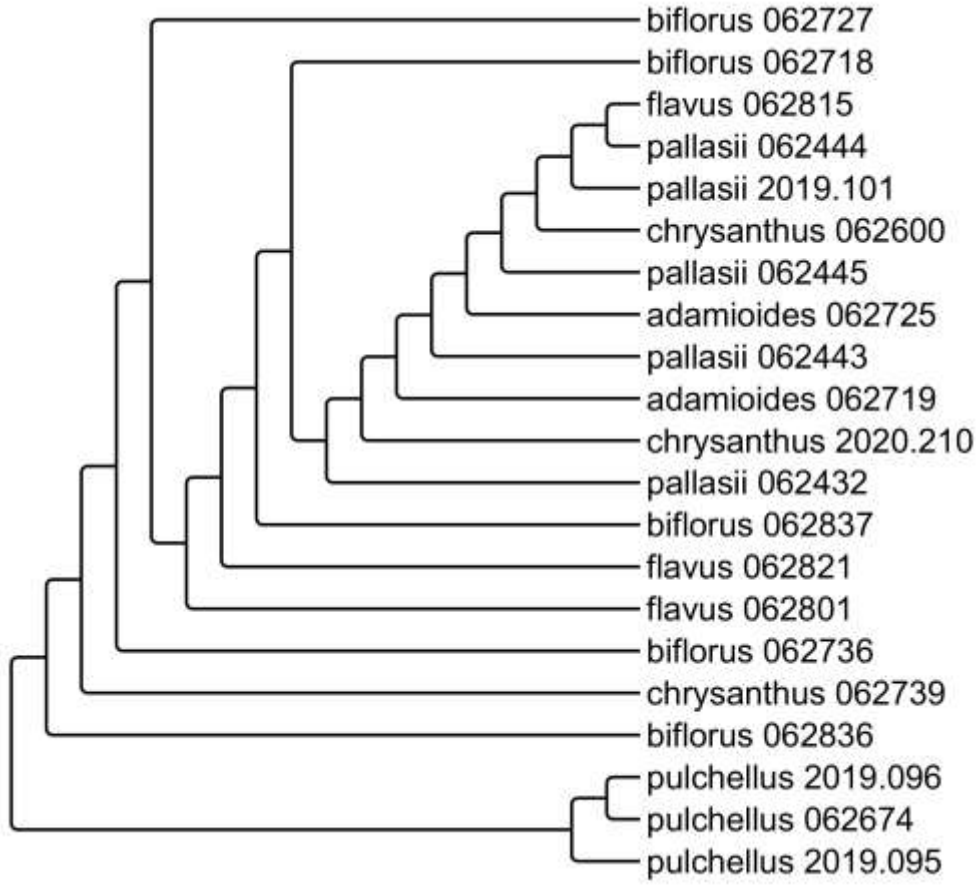

Figure 7. UPGMA dendrogram, demonstrating the phylogenetic relationships between the examined populations of genus *Crocus*

4. CONCLUSIONS

The examined superoxide dismutase polymorphism found by us in the studied species of the genus *Crocus* gives us reason to summarize that this enzyme is a suitable marker both for the analysis of intraspecific heterogeneity and for interspecific comparisons.

ACKNOWLEDGMENTS

This work was financially supported by the National Science Fund, Ministry of Education and Science, Bulgaria (Project "Biodiversity and taxonomic structure of Iridaceae Juss. In Bulgarian flora" number KP-06-N31/5). The authors thank Ivan Kostadinov and Vladimir Trifonov for the collected plant material.

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