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GENETIC DIVERSITY AND MOLECULAR TAXONOMY STUDY OF THREE GENERA FROM *IRIDACEAE* FAMILY IN THE BULGARIAN FLORA BASED ON ISSR MARKERS

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ABSTRACT

Iridaceae is a family of perennial plants with almost worldwide distribution. The taxonomy of the family is based mainly on the morphology, anatomy, embryology and chromosome numbers. The systematics and phylogeny within the family is still subject of debates. This is mainly because most of the classification schemes and determination keys are based on morphological descriptions. These features are often unreliable for exact determination of the species with convergent morphology but inhabiting different ecological localities. In Bulgaria the family is represented by four genera. The genetic diversity and the relations between seven Bulgarian species from the Iridaceae family were examined by ISSR markers. The ISSR-PCR reactions were carried out with seven different ISSR primers and genomic DNA isolated from 13 samples. The distribution of polymorphic PCR products was analyzed by PAST software. The combined results of genetic variants were used to construct a consequent unrooted diagram.

The obtained results clearly defined the two subfamilies in Iridaceae family – Iridoideae and Crocoideae Burnett. The observed grouping of studied species did not coincide with the classification schemes based on morphology features, but was in agreement with the phylogenic studies. Our data confirmed the hypothesis for the polyphyletic origin of species from subgenus Limniris (Tausch) Spach – ser. Laevigatae (Diels) Lawrence and showed their genetic similarity to subgenus Iris ser. Iris. This work, to our best knowledge, is the first attempt to reassess by means of molecular markers the entire taxonomical scheme of the recent members of Iridaceae in Bulgaria.

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Keywords: *Iridaceae*, ISSR markers, molecular taxonomy, phylogeny

Introduction

Iridaceae (Juss.) is a family of perennial, rhizomatous or bulbous plants with almost worldwide distribution that includes more than 2000 species. They inhabit diverse natural habitats, exhibit high adaptability and wide variability of their physiological and morphological features, which makes investigations of their taxonomy, evolutionary history and the phylogenic relations a serious challenge (10, 14).

The leaves are found both at the base and on the stem, usually alternate, with the blade oriented parallel to the stem and thus sheathing it at the base. The flowers are either actinomorphic or zygomorphic. Almost all the parts are in threes, starting with two equal whorls of three usually large and showy petal-like tepals, distinct or fused in a tube.

The family has been accepted as a separate taxon in all major classification systems of the 20th century. However, according to Cronquist (6) it was part of the order *Liliales*, while Takhtajan (26) placed it in an order *Iridales*. The Angiosperm Phylogeny Group (1, 2) system based on molecular phylogeny data placed *Iridaceae* in the order *Asparagales*.

The taxonomy of the family is based mainly on the morphology, anatomy, embryology and chromosome numbers (10, 11, 14). Up to 66 genera have been identified in the family worldwide. In Bulgaria the family is represented by four genera: *Romulea* is monotypic, while the genus *Iris* consists of 10 species, *Gladiolus* of 5 species and *Crocus* of 9 species (27).

The systematics and phylogeny within the family is still subject of debates. This is mainly because most of the classification schemes and determination keys are based on morphological descriptions. The genera of the *Iridaceae* family however, usually comprise a limited number of species with quite heterogeneous morphology and hence with a limited number of morphological features suitable for taxonomy purposes. These features are often unreliable for exact determination of the species with convergent morphology but inhabiting localities with different ecological conditions.

For instance the systematics of the species in genus *Iris* L. is problematic. Different researchers who have studied the genus have usually used different determination features and hence proposed different taxonomy schemes (20). The commonly used criteria in the classification of *Iris* are based on morphological, anatomical, ecological features and cytogenetic analyses (7, 8, 9, 20, 23, 35) but they are not reliable (10, 14). The newest phylogenetic schemes in the family are based

on molecular methods (22, 25, 32). However, most of the investigations included taxa distributed in Africa, Australia (11, 12), America (28, 29, 30, 31) and Asia. Most of these species are not represented on the Balkans. The European species and especially the Balkan endemits have not been studied with modern molecular techniques yet.

A new PCR-based molecular marker approach known as inter-simple sequence repeat (ISSR) became available in 1994 (37). ISSRs are semiarbitrary markers and PCR amplification is done using one 16-18 bp primer complementary to a target microsatellite. The primers are composed of a repeated sequence and can be flanked by 2-4 arbitrary nucleotides – anchored primers, at the 3' or 5' end (37). This technique does not require genome sequence information and yields multilocus and highly polymorphic patterns (21, 37). Each band corresponds to a DNA sequence flanked by two inverted microsatellites. The applicability of the ISSR for taxonomic studies has been tested for a number of taxa and taxonomically significant results have been obtained. ISSR technique has been proven suitable for distinguishing between closely related species (4) and even between different populations (5, 24, 36).

This work is, to our best knowledge, the first attempt to reassess by means of molecular markers the entire taxonomical scheme of the recent members of *Iridaceae* in Bulgaria.

The aims of this study were: 1) to select primers that will allow to discriminate taxa with different taxonomical range (genera, species and subspecies) within *Iridaceae*; and 2) to study the genetic diversity and the phylogenetic relationships between the three main genera (*Iris* L., *Gladiolus* L. and *Crocus* L.) of the *Iridaceae* family represented in Bulgaria.

Materials and Methods

Plant material and vouchers

The plant materials used for this study were collected from Northern Greece, the FYR of Macedonia and different floristic regions of Bulgaria, during the 2010 vegetative season. Vouchers specimens of seven different species were deposed at the herbarium of Agricultural University – Plovdiv, Bulgaria – SOA (**Table 1**). Taxonomical delimitation of samples was determined by existing floristic sources and comparative materials in the herbarium collections of SOA, SO and SOM. Fresh samples from each specimen were used for molecular biology studies in the Laboratory of Molecular markers at the Department of Plant Physiology and Molecular Biology, University of Plovdiv.

DNA preparation

Fresh leaves from the collected plants were frozen in pre-cooled with liquid nitrogen mortar and pestle and grinded to fine powder, of which 100 mg was transferred immediately into a pre-cooled microcentrifuge tube for DNA extraction by DNeasy plant mini kit (Qiagen cat. No 69104) following the original protocol.

TABLE 1

List of voucher specimens of the studied species from the *Iridaceae* family

A. *Gladiolus italicus* Mill.
[BG] Balkan Foothill: 35TLH65. Balinovtsi, 511 m, 2010-05-29 (TR & KS), SOA s/n, IR10-0005 (***13***), IR10-0051 (***12***)
B. *Crocus flavus* Haw.
[BG] Rhodopes: 35TMF19. Kobilino, 446 m, 2010-06-07 (TR), SOA s/n, IR10-0021 (***11***); 35TMF20. Kamilski dol, 465 m, 2010-06-07 (TR), SOA s/n, IR10-0041 (***10***)
C. *Iris pseudacorus* L.
[BG] Black Sea Coast: 35TNH59. Arkutino, 4 m, 2010-05-16 (TR & KS), SOA s/n; IR00001 (***14***); 35TNJ80. Батова, 27 m, 2010-05-16 (TR & KS), SOA s/n, IR00002 (***4***);
[BG] Tracian Plain: 35TLG29. Suhozem, 248 m, 2010-05-02 (T.Hristeva), SOA s/n, IR00005 (***6***); 35TKG77. Zvanichevo, 226 m, 2010-05-30 (KS); SOA s/n, IR10-0010 (***5***)
[MK] Macedonia: 34TFL48. Bansko, 228 m, 2010-05-08 (KS), SOA s/n, MK-0019 (***15***)
D. *Iris reichenbachii* Heuff.
[GR] Greece: 35TMF05. Nea Santa, 615 m, 2010-04-24 (TR & KS), SOA s/n, GR-003 (***8***)
[MK] Macedonia: 34TEM92. Shtip, 300 m 2010-05-06 (I.Denev), SOA s/n, MK-0010 (***9***)
E. *Iris germanica* L.
[BG] Black Sea Coast: 35TPJ21. Tyulenovo, 1 m, 2010-05-07 (TR & KS), SOA s/n, IR00004 (***2***)
[MK] Macedonia; 34TEM70. Vodovrati, 398 m, 2010-05-07 (KS), SOA s/n, MK-0007 (***1***)
F. *Iris pumila* L.
[BG] Black Sea Region: Yaylata, 13 m, 2010-05-17 (TR), SOA s/n, IR-00003 (***7***)
G. *Iris sintenisii* Janka
[BG] Rhodopes: 35TMG00, Chuchuliga, 610 m, 2010-06-07 (TR), SOA s/n, IR10-0020 (***3***)

Legend: [Country code] region (*in bold*), MGRS coordinates, nearest toponym, altitude, date (collector), herbarium, sample temporary numbers (*lane number*)

The absorption at 260 nm was used to determine concentrations of the isolated DNA samples, while the ratios A260/A280 и A260/A230 to determine presence of contaminations like proteins, polyphenolic compounds, sugars and lipids. The average amounts of isolated DNA were 250-300 ng and the above counted contaminations were present in negligible amounts.

Primers

Seven ISSR primers from Primer Set #9 (University of British Columbia, Nucleic Acid-Protein Service Unit, NAPS Unit, http://www.michaelsmith.ubc.ca/services/NAPS/Primer_

Sets/) were tested. Because the production of Primer Set #9 was discontinued by UBC–NAPS Unit, the primers were ordered from Metabion International AG, Martinsried, Germany and upon arrival were dissolved in DNase-free water to 100 mmol final concentration.

ISSR-PCR reaction conditions

Approximately 150 ng (2 µL) DNA template was taken for each sample and mixed in 200 µL PCR tube with 1 µL primer (100 mmol.L^{-1} concentration), 25 µL PCR master mix (Fermentas, Cat No. K0171) and 22 µL DNase-free water (supplied with the master mix kit). The PCR tubes were placed in TC-512 THERMAL CYCLER (Techne) PCR apparatus and PCR amplification was carried-out by using the following program: initial DNA melting at 94°C – 5 min; next 35 cycles at 94°C – 1 min; 43/55°C – 1 min 30 s; 72°C – 2 min 30 s and final extension at 72°C for 6 min. The PCR products were mixed with 7.5 mL of loading dye (Fermentas #R0611), loaded onto 1.5% agarose gel containing 0.5 mg.mL^{-1} ethidium bromide (final concentration) covered with 1X TAE buffer and separated by applying 3.5 volts per cm electrical current. The size of the products was determined by comparison with a DNA ladder (Fermentas GeneRuler#SM0311). The PCR products were visualized by UV light.

Data analysis

The gel images were captured by BIO-VISION+3026.WL system (Vilber Lourmat) using four different exposition times and processed by the accompanying software. The amplified unambiguous bands were scored by molecular masses using the GelPro Analyzer software. Next they were manually allocated into classes of molecular weights for completion of Boolean matrices for the presence/absence (0/1) of bands with the results of each primer.

The binary data were used to construct rectangular matrices using the PAST ver. 1.89 computer software (15) from the data for each gel exposition. The distances obtained from all images were recalculated to average distances for each primer. All the average matrices were summarized to a consequent distance matrix. The results based on genetic distances of the studied species were used to construct a consequent unrooted tree by the T-Rex 3.0a1 software (Vladimir Makarenkov, University of Quebec in Montreal), using the Unweighted neighbor-joining method. The dendrogram was plotted by the PhyloDraw software ver. 0.82.

Results and Discussion

Initially we tested the ability of the different ISSR primers to successfully amplify polymorphic PCR products using various PCR conditions. We were able to select 7 ISSR primers that produced polymorphic bands suitable for distinguishing sections and genera. Four of the primers were with single 3'-arbitrary nucleotide: p810, p811, p817 and p826, and three with two 3'-arbitrary nucleotides: p7, p836 and p857 (**Table 2**). The products of all seven primers showed clustering that coincided with the accepted taxonomic scheme for the species in the *Iridaceae* family (**Fig. 1**). Optimal amplification was achieved at annealing temperature of 55°C.

TABLE 2

ISSR primers used in this study and their specifications

Primer	Primer sequence	Number of polymorphic bands	Range of the amplified bands, (min-max size in bp)
p7	**$(AC)_8$GA**	21	100-1113
p810	**$(GA)_8$T**	22	78-1306
p811	**$(GA)_8$C**	23	93-1436
P817	**$(CA)_8$A**	28	82-1490
P826	**$(AC)_8$C**	19	92-1120
P836	**$(AG)_8$YA**	18	128-1362
P857	**$(AC)_8$YG**	26	388-3400

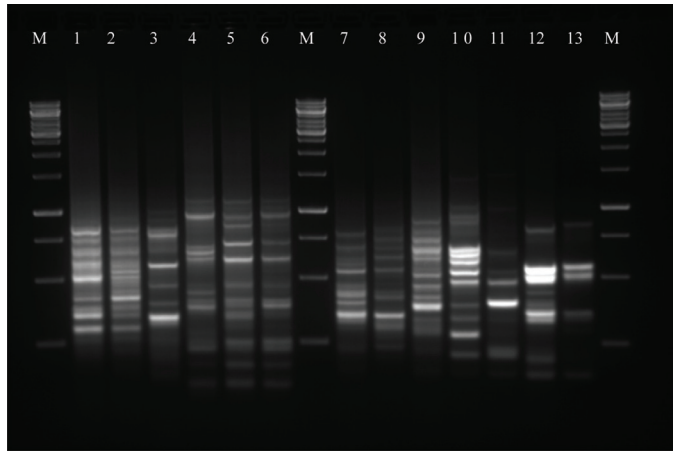

Fig. 1. ISSR-PCR amplification profile with primer 817. Samples from left to right: **1**, **2** – *Iris germanica*; **3** – *I. sintenisii*; **4**, **5**, **6** – *I. pseudacorus*; **7** – *I. pumila*; **8**, **9** – *I. reichenbachii*; **10**, **11** - *Crocus flavus*; **12**, **13** – *Gladiolus italicus*); **M** – molecular weight standard.

The obtained data from ISSR-PCR reactions with each of the seven primers were combined and used to build a consequent unrooted tree. The resulting diagram displayed a definitive separation of the studied species between two distinct clades (**Fig. 2**). The most similar species from the *Iris* genus were clustered in the clade (C-G). The second clade (A-B) comprised the bulbous species *Gladiolus italicus* Mill. (A) and *Crocus flavus* Haw. (B).

The results obtained suggested that the representatives of the two genera are quite different in their morphological features but are probably closer on a genetic level, which separated them from the genus *Iris*. Such grouping is in agreement with the classification proposed by Goldblatt and Manning (13). The authors grouped the genera *Gladiolus* and *Grocus* in a subfamily *Grocoideae* (**Fig. 2A-B**).

The first taxonomy scheme of the genus *Iris* was proposed by Dykes (9). It was later modified and supplemented by Lawrence (18) and Rodionenko (23). However the variability in commonly used morphological, anatomical, ecological features is a prerequisite for alternate taxonomical decisions (3).

According to the classification scheme proposed by Mathew (19) the *Iris* species in Europe belong to two subgenera: subgenus *Iris* (perygonial leaflets with fringes) and subgenus *Limniris* (Tausch) Spach (perygonial leaflets without fringes).

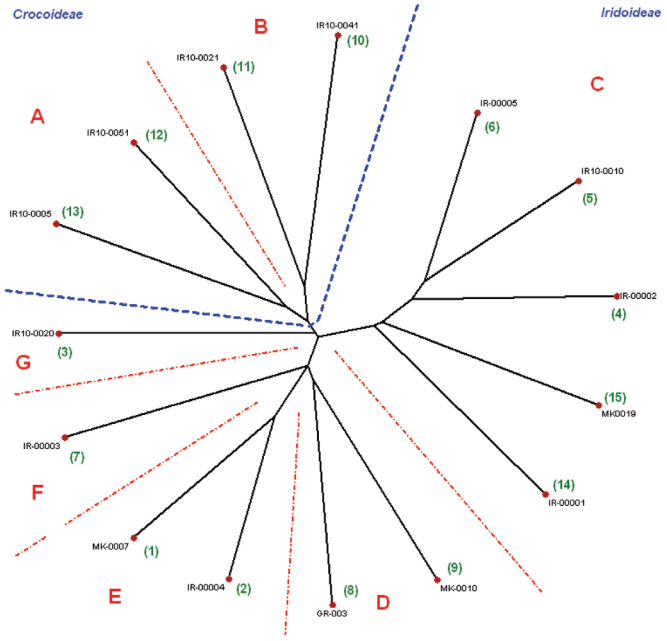

Fig. 2. Final consequent unrooted tree built by stacking of the Euclidean distances using the results of 7 ISSR markers. The numbers in brackets are the same as in **Fig. 1** and **Table 1**. **A** – *Gladiolus italicus*, **B** – *Crocus flavus*, **C** – *Iris pseudacorus*, **D** – *I. reichenbachii*, **E** – *I. germanica*, **F** – *I. pumila*, **G** – *I. sintenisii*.

The clustering in the clade (C-G) in the consequent diagram (**Fig. 2**) displayed a clear division in the clade of genus *Iris* into two subclades. The first one comprised *I. sintenisii* Janka (**Fig. 2G**). The second subclade (**C-F**) united four morphologically different species *I. pseudacorus* L., *I. reichenbachii* Heuff., *I. pumila* L. and *I. germanica* L. Among the members of this subclade the populations of *I. reichenbachii* showed high similarity despite the fact that they were collected from different countries – Greece and the FYR of Macedonia. Such similarity was probably due to similarities on the genetic level and could be used for clear differentiation of the populations of this species from others (**Fig. 2D**). *I. pumila* (F) and *I. germanica* (E) displayed a relatively small difference from *I. reichenbachii*, which can be a clue for the existence of a close relationship between these species.

Iris germanica have been considered a natural hybrid between *Iris pallida* Lam. and *Iris variegata* L. In 1889 it had additionally hybridized with a horticultural Mediterranean species of *Iris* (16, 34). Therefore it is considered a species with hybrid origin – *Iris* × *conglomerata* NC Hend (17). It is very probable that the wild populations of *I. germanica* are in fact representatives of *I.* × *conglomerata*. *Iris germanica* was suggested to be a grandparent of the recent species with fringed perygon leaflets. Probably this is the reason why *I. germanica* samples took an intermediate position in our consequent diagram.

According to the morphology-based classifications, the species without fringed perygonal leaflets are grouped in the subgenus *Limniris* sect. *Limniris*. With the exception of one species, the members of sect. *Limniris* are representative of the Asian and North American flora. Some Asian species were subject of secondary introduction in Europe. This subgenus was represented in our study by two species: *I. sintenisii* (G) and *I. pseudacorus* (C). Our data are in agreement with the hypothesis for the polyphyletic origin of *Limniris* (32, 33). *Iris pseudacorus* belongs to ser. *Laevigatae* (Diels) Lawrence and in spite of the similar morphology occupies a divergent position from *I. sintenisii* – sect. *Spuriae* (Diels) Lawrence. Despite the fact that *Iris pseudacorus* is one of the widely spread species from this group, the samples collected form different locations did not display significant genetic differences.

Conclusions

The study demonstrated the selectiveness of the ISSR markers as an option to resolve the taxonomical problems in *Iridaceae*. This study confirms the polyphyletic origin of subgen. *Limniris* and the monophyletic character of the subfamilies *Crocoideae* and *Iridoideae*.

In general the observed grouping in the consequent diagram of the studied species often did not coincide with the classification schemes based on morphology features, but was in agreement with the modern phylogenic studies. Therefore we are planning to continue and expand the investigations of this group based on molecular markers in order to update and revise the existing taxonomical scheme and to assess the phylogenic relationships between the *Iridaceae* species represented on the Balkans.

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