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 phylogenetic 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.


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 phylogenetic 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 endemics 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 deposited 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 grinding 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.

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

<table>
<thead>
<tr>
<th>A. <em>Gladiolus italicus</em> Mill.</th>
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<tr>
<td><strong>[BG]</strong> Balkan Foothill: 35TLH65. Balinovtsi, 511 m, 2010-05-29 (TR &amp; KS), SOA s/n, IR10-0005 (13), IR10-0051 (12)</td>
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<th>B. <em>Crocus flavus</em> Haw.</th>
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<tr>
<td><strong>[BG]</strong> 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)</td>
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<th>C. <em>Iris pseudacorus</em> L.</th>
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<tr>
<td><strong>[BG]</strong> 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)</td>
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<th>D. <em>Iris reichenbachii</em> Heuff.</th>
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<tr>
<td><strong>[GR]</strong> Greece: 35TMF05. Nea Santa, 615 m, 2010-04-24 (TR &amp; KS), SOA s/n, GR-003 (8)</td>
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<th>E. <em>Iris germanica</em> L.</th>
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<tr>
<td><strong>[MK]</strong> Macedonia: 34TEG42. Vodovrati, 398 m, 2010-05-07 (KS), SOA s/n, MK-0021 (13)</td>
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<th>F. <em>Iris pumila</em> L.</th>
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<td><strong>[BG]</strong> Black Sea Coast: 35TPJ21. Tyulenovo, 1 m, 2010-05-07 (TR &amp; KS), SOA s/n, IR00004 (2)</td>
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<tr>
<th>G. <em>Iris sintenisii</em> Janka</th>
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<tr>
<td><strong>[BG]</strong> Rhodopes: 35TMG00. Chuchuliga, 610 m, 2010-06-07 (TR), SOA s/n, IR10-0020 (3)</td>
</tr>
</tbody>
</table>

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 and 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_
initially we tested the ability of the different iSSR primers to

### Results and Discussion

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 *Grocioideae* (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 (perygonal 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. 2G) 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 phylogenetic 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 phylogenetic relationships between the Iridaceae species represented on the Balkans.

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