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Molecular authentication of some rare *Iris* (Iridaceae) species from Uzbekistan

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Abstract

The determination of nucleotide sequences in DNA, sequencing of chloroplast genomes techniques, based on the use of combinations a significant number genetic markers, as well as the nuclear genome are widely use for inventory of rare plant species, ecological monitoring and species diversity assessment. The results of our molecular analysis are used to examine the taxonomic identity and phylogenetic relationship of the studied *Iris* species collected in Uzbekistan. The genotyping of three-four functional loci of nrDNA and cpDNA was performed for efficient species identification and confident results. The nuclear *ITS* gene sequences and chloroplast *trnL-trnF, rbcL, matK* gene sequences were obtained from 10 *Iris* species and have proven to be useful as molecular markers for species identification. Sequences of cpDNA regions, have been used to assess interspecific relationships of *Iris.* Hierarchical clustering were constructed using 13 consensus *matK* gene sequences. In this study, we consider the possibility of successful application the DNA barcoding as a tool for assessment of wild *Iris* species diversity in Uzbekistan.

Keywords

biodiversity, DNA barcodes, *Iris*, *ITS*, *matK*, *rbcL*, *trnL-F*

Introduction

The Quaternary period in Asia saw significant climate fluctuations that had a profound impact on the evolution and distribution of plant life, resulting in notable genetic outcomes and increased speciation activity. Thus, the flora of Central Asia includes 9520 species and characterized by an extensive range of plant species with 20% endemics. At the same time, the flora of Uzbekistan includes approximately 4385 species of vascular plants, 1/5 of the entire flora is monocotyledonous plants (1, 2). Also, it is characterized by the plenty of ornamental geophytes, widely used in horticulture due to their exceptional decorativeness. The *Iridaceae* family is represented by bulbous and root-like plants.

Flora of Uzbekistan is distinguished by its abundant diversity of species (3,4). It is one of the center early diversification of monocotyledonous geophytes, significantly surpassing other geographical areas in terms of the absolute number of endemics. These include a specific group of relic endemic plants, which are nearly to disappear off the face of the earth (5). So, there is an extremely high risk losing a unique species and their genetic information. Therefore, the purpose of our research is a species diversity inventory of the flora of Uzbekistan using the DNA barcoding method.

The relevance of this study is determined by the significant interest in to the preservation of the studied group. The rare taxonomically important *Iris* species growing in Uzbekistan territory were chosen as objects of this study.

This article explores the use of DNA barcoding as a technique for evaluating the plant diversity on representatives of *Iris* genus wildly growing in Uzbekistan. The originality of individual plant communities and various biogeographical regions characterizes the territory.

DNA barcoding is a method based on the sequencing of variable taxonomically significant DNA sequences. This technology is a necessary tool for the accurate species identification and systematization of genetic resources, as well as effective technique for the inventory of rare and endangered species, that allows the screening and assessing species diversity with preservation of specimens in their natural habitat.

For species identification, the universal DNA barcodes with a small fragment are the most effective (6, 7). There hasn't been enough focus on molecular analysis of the studied objects inhabiting this area. Therefore, this work is justified for the study of the taxonomic diversity of the identified objects. The *Iridaceae* family, according to APG IV (8), includes 66 genera and more than 2244 worldwide species belonging to petaloid monocot plants (9). There has been insufficient investigation in the genetic diversity of *Iridaceae* within the flora of Uzbekistan at different taxonomic levels (generic, interspecific) using DNA markers. In recent years, based on modern molecular phylogenetic studies, the classification of this family has changed dramatically (10). The typical representative of this family is *Iris L.* genus, which is the original group of plants in morphological, systematic, phylogenetic, biological, ecological, geographical and other properties (11, 12, 13). Many irises are economically important ornamental plants, that have been in cultivation for a long time for their beautiful flowers, as well as medicinally valuable plants possessed an unusually wide range of biologically active substances, including flavonoid, phenolic, alkaloid compounds possessing insecticidal properties which could effectively substitute chemical preparations (14, 15).

Materials and Methods

Materials sampling .

As model objects the endemic and rare *Iris* Tourn. ex L. (Iridaceae Juss.) species were used in this study. The materials used for this research were wild plant species collected from various geographical regions of Uzbekistan, mountainous and foothill areas with rich biological diversity (16). Three samples of each taxa and three samples for each herbarium specimen were taken for the genetic analysis.

For analyzes we used silica gel dried leaf tissues and herbarium tissues of specimens in National Herbarium (Institute of Botany, Tashkent). For the reconstruction of intragenus topology, sequences of three taxa were taken from GenBank (https://www.ncbi.nlm.nih.gov). The nomenclature of sampled species, subgenera and sectional classification are given (11) (Table 1).

DNA extraction, amplification and sequencing

Total DNA was extracted using *GeneJET Plant Genomic DNA Purification Kit*, with some modifications for herbarium specimens. The quality and the quantity of nucleic acids DNA was assessed spectrophotometrically. *DNA barcoding* technique is an actual molecular genetic method

Table 1. Subgeneric representatives of the genus *Iris* L. growing in Uzbekistan

No	Taxa, collection year	Distribution in Uzbekistan, coordinates, rarity status
	Subgenus *Iris* B. Mathew	
	Section *Iris* L.	
1	*Iris alberti* Regel	South foothills of the Chatkal ridge, Kumbel Pass
	Section *Regelia* (Foster) Foster	
2	*Iris korolkowii* Regel* (2011)	Western Tien-Shan, South-Eastern Pamir-Alay: Turkestan, Malguzar, Zeravschan, Hissar, Kuhitang, Babatag ridges: Karzhantau, Ugam, Pskem, Chatkal, Kuramin ridges. Collected in Kuramin ridge, Sarytash river valley, 2227 m. 41°06'34"N 70°37'16"E Endemic of the Central Asia
3	*Iris stolonifera* Maxim.	Pamir-Alay: Zeravschan, Hissar, Babatag, Kuhitang, Kuhistan, Hissar-Darvaz, Panzh region
	Section *Hexapogon* (Bunge) Baker	
4	*Iris longiscapa* Ledeb.	Kyzylkum, Mirzachul, Karshy-Karnabchul steppe, Surkhan-Sherabad valley, Nuratau, Zirabulak-Ziadin mountains, Western Hissar, South Aral Sea, Syrdarya region
5	*Iris falcifolia* Bunge	Kyzylkum, Mirzachul, Karshy steppe, Karnabchul, Surkhan-Sherabad valley, foothills of Hissar, Kuhitang, Babatag ridges
	Section *Monolepis* (Rodion.) B. Mathew	
6	*Iris kolpakowskiana* Regel	North- Western Tien-Shan: Karzhantau, Chatkal, Kuramin, Ugam, Pskem ridges, Chimgan pass
7	*Iris winkleri* Regel	Tien-Shan, North Pamir-Alay: Chatkal, Kuramin ridges, Angrensay Status 2. Very rare species of the western Tien-Shan

	Subgenus *Limniris* (Tausch) Spach em.Rodion.	
	Section *Sclerosiphon* (Nevski) Sennikov & F.O.Khass.	
8	*Iris songarica* Schrenk ex Fisch. & C.A.Mey.	Kyzylkum, Nuratau, Karshy steppe, Karnabchul, Fergana valley; foothills of the Zeravschan, Western Tien Shan: Aktau, Zirabulak-Ziadin mountains, Kuhiston, Western Hissar, Kuhitang ridges; Southern Aral Sea, Ustyurt region
	Section *Tenuifoliae* (Diels) Doronkin	
9	*Iris loczyi* Kanitz	Western Tien-Shan: Chatkal, Kuramin, Alay, Zeravschan, Western Hissar, Kuhitang ridges; Hissar-Darvaz region
	Section *Xyridion* Tausch	
10	*Iris sogdiana* Bunge* =*Iris spuria* L. (2019)	Western Tien-Shan: Low Zeravschan, Kashkadarya, Sangardak-Tupalang, Baysun regions; Chimgan, Hissar, Kitab, Surkhan, Ugam-Chatkal reserves. Collected in the Tashkent Alatau, 1900 m. 41°30'48.9"N 70°02'08.9"E
	Section *Haloiris* Doronkin	
11	*Iris lactea* Pall.	Tien-Shan, North Pamir-Alay: Fergana valley, Kayrakkum region; Alay, Zeravschan ridges
	Subgenus *Scorpiris* Spach	
	Section *Juno* (Tratt.) Maxim	
12	*Iris bucharica* (Foster) Vved.	South slope Hissar ridge, Baysun mountains, Kuhitang, Sangardak
13	*Iris hippolyti* (Vved.) Kamelin*(2015)	South-Western Kyzylkum: Navoi region: Kokchatau mountains, Collected near Zafarabad village. 450 m. 40°20'49"N 67°33'50"E Status 1. Extremal rare narrow-local endemic of the remnant low mountains of Kyzylkum (Kokchatau)
14	*Iris svetlanae* (Vved.) T.Hall & Seisums*(2012)	Western Pamir-Alay: Kashkadarya river basin, western spurs of the Hissar and Zeravschan ridges. Collected in the Dehkanabad vicinity, Tally pass. 1300 m. 38°08'23.2"N 66°31'59.8"E Status 2**.** Rare endemic of the western Pamir-Alay
15	*Iris vicaria* (Vved.) T.Hall et Seisums*(2014)	South-Western Pamir-Alay: Hissar, Kuhitang, Babatag ridges, Baysun mountains, Saukbulak mountain Collected in the South-Western Hissar, Baysuntau, Amankhon vicinity. 1431m. 38°13'35.0"N 67°18'03.0"E Endemic of the South-Western Pamir-Alay
16	*Iris austrotschatkalica* Tojibaev, F. Karim, et Turginov	Fergana valley, South foothills of the Chatkal ridge
17	*Iris albomarginata* R.C.Foster	Western Tian-Shan: Pskem valley to lake Mahbalkul
18	*Iris chrysopetala* Sennikov, F.O.Khass. & Pulatov	Surhandarya Region: Babatag ridge
19	*Iris khassanovii* Tojibaev & Turginov	Southern Pamir-Alay, South slope of Hissar ridge, Baysun mountains
20	*Iris linifolia* (Regel) O.Fedtsch.	Western Tien–Shan: Kuramin ridge, Pamir-Alay: Alay ridge, Angren
21	*Iris magnifica* (Vved.) F.O. Khass. & Rakhimova*(2014)	Pamir-Alay: Samarkand mountains, North slope of the Zeravschan ridge, Agalyk Aksay, Mi-rankul, Akdarya, Amankutan, Tersaksay, Urgutsay natural boundaries, Sangtud and Takhta-Karacha pass. Collected in Samarkand region, Urgut district, Amankutan pass. 1200 m. 39°18'53.6"N 66° 58'36.3"E Status 2**.** Rare endemic of the Zeravschan ridge
22	*Iris maracandica* (Vved.) Wendelbo	North-Western Pamir-Alay: Zeravschan, Malguzar, Nuratau, Aktau, Urgut, Zirabulak-Ziadin mountains
23	*Iris narbutii* O.Fedtsch.	Western Tien-Shan: Tashkent region, Mogoltau, Pamir-Alay: Nuratau, Aktau
24	*Iris orchioides* Carrière*(2020)	Western Tien-Shan: Karzhantau, Ugam, Pskem, Maydantal, Chatkal, Kuramin ridges. Collected in the western slopes of the Chatkal ridge, Great Chimgan 2238m. 41°30'29.8"N 70° 01'02.8"E Status 3**.** Rare endemic of the western Tien-Shan
25	*Iris pseudocapnoides* Rukšāns	Western Tien-Shan: Great Chimgan mountain, Chatkal
26	*Iris capnoides* (Vved.) T. Hall & Seisums	Western Tien-Shan: Kuramin ridges, Chatkal, Yangiabad
27	*Iris parvula* (Vved.) Sennikov	Western Pamir-Alay: Turkestan, Zeravschan, Hissar, Baysun mountains

28	*Iris tubergeniana* Foster*(1994)	Western Tien-Shan: Tashkent Alatau, Karzhantau, Ugham, Chatkal ridges. Collected in Karzhantau foothills, Tashkent region, Kibray district, village Mayskiy 574 m. 41°27'31"N 70°01'02.8"E Endemic of the western Tien-Shan
29	*Iris vvedenskyi* Nevski ex Woronow & Popov	Pamir-Alay: Kuhitang ridge
30	*Iris warleyensis* Foster*(2019)	Western Pamir-Alay: Zeravschan, Hissar ridges, Kuhitang, Baysun mountains, Takhta-Karacha pass. Collected in the Zeravschan ridge 1871 m. 39°18'36.2"N 66°53'47.5"E Endemic of the western Pamir-Alay
31	*Iris tadshikorum* (Vved.) Vved.	Pamir-Alay: Zeravschan, Urgut, Nuratau, Hissar ridges, Pass between Kalta-Kul and Tashkurgan, Kulsay
32	*Iris linifoliiformis* (Khalk.) Tojibaev & Turginov	Northern Pamir-Alay: Pamir-Alay ridges, Shakhimardan
33	*Iris narynensis* O.Fedtsch.	Tian-Shan: Chatkal Ridge, Alay Ridge, Fergana valley, foothills of the Alay ridge, Nanay
34	*Iris petri* F.O.Khass., Rakhimova & Achilova	Southern Pamir-Alay: Western Hissar: Baysun mountains, Kelif-Sherabad range
35	*Iris rudolphii* F.O.Khass., Esankulov & Achilova	Southern Pamir-Alay. Western Hissar: Surkhan-Sherabad valley, Baysun, Sangardak-Tupalang, Kuhitang, Babatag mountains, Aktash
36	*Iris rodionenkoi* (Lazkov & Naumenko) T.Hall	Western Tien-Shan: Fergana Range, Uygursay
37	*Iris subdecolorata* (Vved.) F.O. Khass. & Rakhimova	North- Western Tien-Shan: Chimgan, Tashkent vicinity, Mogoltau, Zailiy Alatau
38	*Iris victoris* F.O.Khass., Khuzhan. & Rakhimova*(2013)	Southern Pamir-Alay: Western-Hissar, Surkhan-Sherabad valley, Kelif-Sherabad Ridge. Collected in Aktash village 560 m. 37°33'08.0"N 66°41'29.0"E Status 1. Extremely rare endemic of the southern Pamir-Alay
	Section *Physocaulon* (Rodion.) Mathew &Wendelbo **Subsection** *Rosenbachianae* (Rodion.) Sennikov	
39	*Iris nicolai* (Vved.) Vved.	Surhandarya river basin, South slope of the Hissar ridge, Baysun mountains
40	*Iris rosenbachiana* Regel	Hissar ridge: Maydanak mountains

*- the species used in this work

for plant species identification. In this pilot research we applied significant DNA markers: Internal transcribed spacer region of nuclear ribosomal DNA and 3 chloroplast DNA *rbc*L, *matK*, *trnL-F* regions. Sequences of cpDNA non-coding regions are used to assess interspecies relationships and as molecular markers for species identification. They are applying as the core DNA barcodes for vascular plants most extensively, based on the accessibility of the sequencing efficiency and high level of taxonomic resolution (17). cpDNA markers are have proven to be useful as phylogenetic markers for the genus *Iris* (18, 19, 20). The *trnL-trnF* intergenic spacer region between leucine and phenylalanine tRNA genes is used as a barcode DNA for *Iris* genus (21).

PCR amplifications were performed in 25 µL standard reaction mixture containing 2X *Taq* Plus PCR Master Mix with dye (*Applied Biological Materials Inc.,* Canada), 1 µL genomic DNA (10 ng/µL), 1 µL primer. Amplification of the target products was conducted using forward and reverse primer sets (TsingKe, China) on the *C1000 Touch Thermal Cycler (BioRad, USA).* Amplification was carried out for 10 species in three replications. Nucleotide sequences of PCR primers and length of PCR products are given in Table 2.

Table 2. Specific primers used in amplification of marker sequences

Primer names	Sequences	PCR product size	References
ITS1_18S ITS4_26S	5'-TCCGTAGGTGAACCTGCGG-3' 5'-TCCTCCGCTTATTGATATGC-3'	~700 pb	White *et al.* (22)
rbcLa F rbcLa R	5'-ATGTCACCACAAACAGAGACTAAAGC-3' 5'-GTAAAATCAAGTCCACCRCG- 3'	~654 pb	Kress & Erickson (23)
matK-390F matK-1326R	5'-CGATCTATTCATTCAATATTTC-3' 5'-TCTAGCACACGAAAGTCGAAGT-3'	~850 pb	Cuenoud *et. al.* (24)
trnL-F_F trnL-F_R	5'- CGAAATCGGTAGACGCTACG -3' 5'- ATTTGAACTGGTGACACGAG- 3'	~900 bp	Taberlet et al. (25)

PCR conditions had been optimized according to the following parameters (Table 3).

An enzymatic cleanup method with *ExoI* and *SAP* was used for PCR products purification *(Thermo Fisher Sci-*

Table 3. Amplification conditions

Cycling stage	Barcode locus, 25 *mkl*			
	ITS	***matK***	***rbcL***	***trnL-F***
Initial denaturation	94°C – 5 min	95°C – 4 min	94°C - 3 min	94°C - 3 min
Denaturation	94°C - 30 s	94°C - 30 s,	94°C - 30 s	94°C - 45 s
Annealing	50°C - 30 s	53°C - 1 min	54°C - 45 s	50°C - 45 s
Extension	72°C - 45 s	72°C - 1 min	72°C – 45 s	72°C - 1 min
	35 cycles	35 cycles	35 cycles	32 cycles
Final extension	72°C - 10 min	72°C - 10 min	72°C - 10 min	72°C - 8 min
	4°C - ∞	4°C - ∞	4°C - ∞	4°C - ∞

entific, USA). Termination reaction was performed using a commercial kit Brilliant Dye Terminator v3.1 Cycle Sequencing Kit (*Nimagen*, Netherlands). The determination of the nucleotide sequence was performed on an automatic genetic analyzer *ABI 3500 DNA Analyzer* (Applied Biosystems, USA) in a forward and reverse directions (26). Sequence chromatograms were viewed and evaluate in Sequence Scanner 1.0.

Alignment and phylogenetic analyses

The DNA sequences were aligned using BioEdit version 7.0.01. software and saved in FASTA format. The sequence alignment was performed using ClustalW algorithm. The results of hierarchical clustering are presented by a constructed dendrogram obtained by Maximum Likelihood (ML) algorithm, using MEGAX program. The reliability assess of the resulting phylogenetic tree topology was obtained with Bootstrap analysis (1000 replicates) (27). The resulting graphic image was visualized using the FigTree v1.4.0 program. The model was selected using the jModelTest 2.1.10 program (28).

Results and discussion

The genus *Iris* Tourn. ex L. is one of the largest genus in *Iridaceae* Juss. family, *Iridoideae* Eaton subfamily, *Irideae* Kitt. tribe. It comprises approximately 337 accepted taxonomically challenge species by the recent revision of the genus in the world species (29) and the most diversified which are originate in the Northern Hemisphere (30), naturally widespread in the Central Asia (11).

Iris species are considered one of the best decorative plants and also used in various traditional medicines, many of them are shown antioxidant, cytotoxic, antibacterial, anti-inflammatory, antituberculosis properties due to bioactive compounds (31, 14, 15). The represents of uzbek irises are valuable rare and endangered plants with ornamental potential. Their number is constantly decreasing due to habitat loss by various factors. So, they require measures for protection, preservation and distribution as promising spring ornamental plants.

Phylogenetic relationship among taxa on the sections levels within the genus *Iris* are subject of different systematic studies, which carried out based on comparison of classical morphological properties, ontogenesis features, cytogenetic analyses as well as molecular analyses (11, 31-41).

There is still no generally accepted system of irises. The recent studies have shown, the main reasons are an extremely high ecological and genetic plasticity due to the polymorphism of species in natural populations and to the significant hybridization in the genus evolution.

The sections independence is confirmed by diverse morphological features of vegetative organs, leaf development, colorful perianth (sepals and petals) (11). Underground organs as rhizomes, stolons, bulbs or tuberous roots have evolved in *Iris* species. Subgeneric classification mainly base on diagnostic features of storage organs type. They form a two natural group characterized by fleshy storage roots: bulbous and rhizomatous irises (36). Also seed coat structure, type of sepal crests, presence or absence of sepal beards (33) are important morphological characters in the taxonomic delineation within *Iris.* By authors, the genus *Iris* includes six subgenera: *Iris* B. Mathew, *Limniris* (Tausch) Spach, *Nepalensis* (Dykes) Lawrence, *Xiphium* (Miller) Spach, *Scorpiris* Spach, and *Hermodactyloides* Spach (10, 37).

According the last taxonomic treatment, taking into account the recently described taxa, the flora of Central Asia represented by 57 taxa of the genus *Iris*, with the greatest number of endemics occurring in this area, counted 36 species (11). By recent data, Uzbekistan is the most species- rich with *Iris* and treated as one of the largest centers of biodiversity counted 40 taxa (Table 1.). Six species are listed in the “The Red Data Book of Uzbekistan” (Fig. 1) (5).

The genus *Iris* in the flora of Uzbekistan includes three subgenera subdivided into 10 sections (Table 1.). Subgenus *Iris* B.Mathew rhizomatous irises represented by *Iris* L. section with bearded outer tepals, dorsiventral leaf; *Hexapogon* (Bunge) Baker em. Rodion section with isolateral-spongy leaf and close related *Regelia* Lynch section. Subgenus *Limniris* (Tausch) Spach represents rhizomatous, beardless outer tepals irises, forming the section *Limniris* Tausch with unifacial, isolateral-palisade leaves. Species in the genus *Scorpiris* Spach (*Juno* Tratt) is remarkable on morphological features group of bulbous irises with reduced petals, crest and noncrest sepals, it consists of a single section *Juno* (Tratt.) Maxim. (crested/bearded irises) with dorsiventral spongy leaves. The subgenus *Hermodactyloides* Spach (the reticulatas), reticulate-bulbed bulbous irises form the section *Monolepsis* (Rodion.) B. Mathew, characterized by bifacial, channeled

Fig.1. Endangered *Iris* species collected in Uzbekistan: 1- *Iris orchioides;* 2- *Iris hippolyti;* 3- *Iris svetlanae;* 4- *Iris warleyensis;* 5- *Iris magnifica.* Photos by Ortikov E

leaves (37). But, it is difficult to describe the genus *Iris* based on morphological diverse only (38).

As a result of our analysis, 34 nucleotide sequences for 10 species of the genus *Iris* are received and has been submitted in the international Genbank database provided with ID numbers (http://www.ncbi.nlm.nih.gov) (Table 4).

Table 4. Nucleotide sequences of studied species, submitted in the GenBank

Identification numbers in *NCBI*			
ITS	***matK***	***rbcL***	***trnL-F***
Iris orchioides (Carriere) Vved.			
MZ046072.1 754 bp	MZ054594.1 779 bp	MZ054604.1 901 bp	MZ054614.1 863 bp
Iris hippolyti (Vved.) Kamelin			
MZ046073.1 758 bp	MZ054595.1 761 bp	MZ054605.1 896 bp	MZ054615.1 877 bp
Iris svetlanae (Vved.) T.Hall et Seisums			
MZ046074.1 758 bp	OP081804.1 770 bp	MZ054606.1 931 bp	MZ054616.1 850 bp
Iris tubergeniana (Foster) Vved.			
MZ046075.1 756 bp	MZ054597.1 776 bp	MZ054607.1 927 bp	MZ054617.1 861 bp
Iris sogdiana Bunge			
MZ046076.1 441 bp	MZ054598.1 915 bp	MZ054608.1 530 bp	-
Iris vicaria (Vved.) T. Hall & Seisums			
-	MZ054599.1 773 bp	MZ054609.1 943 bp	MZ054618.1 830 bp
Iris warleyensis (Foster) Vved.			
-	MZ054600.1 770 bp	MZ054610.1 875 bp	MZ054619.1 866 bp
Iris korolkowii Regel			
-	MZ054601.1 773 bp	MZ054611.1 906 bp	MZ054620.1 860 bp
Iris magnifica (Vved.) Vved.			
-	MZ054602.1 778 bp	MZ054612.1 900 bp	MZ05462.11 851 bp
Iris victoris F.O.Khass., Khuzhan. et Rakhimova			
-	MZ054603.1 772 bp	MZ054613.1 876 bp	MZ054622.1 866 bp

Thus, three-four locus genotyping was performed for all studied species for maximum efficiency of species identification and high reliability of the results. The DNA barcodes used in this work: *ITS, rbcL, trnL-F, matK* made it possible to successfully species the plants of the genus *Iris* (Table 5).

The table shows the results of genotyping based on four consensus sequences species *Iris hippolyti* (Vved.) Kamelin. A satisfied reproducibility of amplification results is demonstrated for all markers. The identifying reliability of *Iris* with analogous sequences in the NCBI database is at least 92% at genus-level and 100% at species-level. So, the DNA barcodes used in this work: *ITS, rbcL, matK, trnL-F* demonstrate successful, fast and accurate species-level molecular identification.

The current sequences showed the percentage variations in the of Guanine+Cytosine content (% G + C) calculated in the G~C Content Calculator (www.sciencebuddies.org). Thus, in the case of target fragment *matK,* the average content was from 31.3 to 32.2% (Table 6).

nrDNA and cpDNA have proven to be useful and are suggested as phylogenetic markers in the genus *Iris* (10, 18 -20, 33, 38-40). According to literature data the *matK* primer is used successfully for genetic diversity research of the genus *Iris* (20). As noted, using the chloroplast sequence data, the subgeneric classification recognized 6 sub genera and 12 sections within the genus (10).

The *matK* has relatively low evolutionary mutation rates, it is able to provide high level of resolution to understand the interspecific relationships within the genus. Three sequences of the *matK* region used for a phylogenetic dendrogram construction were download from the GenBank database (Fig. 2). The length of the aligned regions of target marker is 741 b.p. Phylogenetic reconstruction based on differences *matK* sequences reflects the evolutionary relationships of 13 taxa within the genus *Iris. Gladiolus italicus* is used as an outgroup, based on previous analysis (20, 33).

The results of phylogenetic analysis using the *matK* region based on the ML algorithm (congruent with NJ) shows the clear genetic division of the *Iris* genus into three major well-supported clades according to 3 subgenera: *Scorpiris, Iris, Limniris*, with a high Bootstrap value. This result confirms by other authors (10) (Fig. 2). The comparative analysis was conducted to reflect interspecific relationships within *Iris* based on the sequence data of *matK* chloroplast genome region. It is revealed 40 variable positions.

In our phylogenetic analysis results subgenus *Iris* comprises two major evolutionary clades. The first clade includes representatives of the most numerous with species section *Juno* (subgenus *Scorpiris): I. bucharica, I. hippolyti, I. svetlanae, I. vicaria, I. magnifica, I. orchioides, I. tubergeniana, I. warleyensis, I. victoris.*

Our analysis resolved, *I. bucharica, I. vicaria* are grouped into a well-supported subclade, that corresponded with “Bucharica group” of previous authors (30, 39). This fact is confirmed by their similar morphology characters with a well developed stem and wingless flower (39). *I. tubergeniana* and *I. orchioides* are placed together into strong supported branch, showing their sister relationship. According authors, they are similar species, completely sympatric, occupying a narrow territory in the Western Tian-Shan (12). A strongly supported separate subclade with a high Bootstrap value- 97, contains closely related species *I. hippolyti, I. svetlanae*, *I. magnifica, I. warleyensis, I. victoris* confirmed by their morphology and geography, mainly having a Pamir-Alay distribution (11). By authors,

Table 5. Identification results of *Iris hippolyti* (Vved.) Kamelin using 4-locus panel

DNA-barcode	Consensus nucleotide sequences (bp)	GenBank accession sequence ID number	Identification results in *BLAST*
ITS	1 caaggtttcc gtaggtgaac ctgcggaagg atcattgtcg ataaccgaac catcggacga 61 cccgagaaca tgtcaacaca acgccgccgc cctcgggcgg cggcaccccc ccatgcggcc 121 cgccggaacg acaccgcctc accccggtgg agcgcgtgcc cgggccggcg cggcggcata 181 acgaaacccc ggcgcggtgg gcgccaagga acactgtata ctcagcgcgg ctgctcccct 241 cacggggagg agccccgcgt acctatcaac aacgttgtac ttctgtacga ctctcggcaa 301 cggatatcta ggctctcgca tcgatgaaga acgtagcgaa atgcgatact tggtgtgaat 361 tgcagaatcc cgtgaaccat cgagtctttg aacgcaagtt gcgcccgagg ccttctggcc 421 gagggcacgc ctgcctgggc gtcacgcctc gtgtcgctcc gcacacatct ttttcccctc 481 tctttaccgg gcggggaggg aggcgtcgtg cggacgcgga gattggccca ccgtgcctcg 541 tgcgcggcgg gccgaagtac gggccgtcgt cgggccgggc gcggcgagtg gtggacgatg (758 bp)	GenBank Sequence ID: MZ046073.1	MH711021.1 *Iris tectorum* partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence Identities: 92.95%
matK	1 tccttcaatg ctggattcaa gatgttcccc ttttacattt cttgcgattc tttcttcgta 61 aatatcataa ttggaatagt tttctcatta ctccgaagaa atctatttat gttttttcaa 121 aagaaaataa aagactattt tggttcctat acaattctta tgtatctgaa tgtgaatttt 181 tattagtttt tcttcgtaaa caatcttctt atttacgatt aacatctttt ggacttttttc 241 ttgagcgaag acatttctat gtaaaaatgg aacatcttca aatgcaacat cttatactta 301 tagtagtatg tcgtgattat tttcaaggaa ccctacggtc cttcaaggat cctttcatgc 361 attatgttcg atgtcaagga aaagcgtttt tggcttcaaa agggactcat tttctgataa 421 agaaatggaa atataatttt gtcaatttat ggcaatatta ttttcacttt tggtatcaat 481 cgtacaggat ccatataaac caattatcaa actattcttt ctatttttgg ggttatcttt 541 caagtttact aaaaaattct tcgacggtaa ggaatcaaat gttagagaat tcatttctaa (761 bp)	GenBank Sequence ID: MZ054595.1	FR870419.1 *Iris hippolyti* chloroplast matK gene for maturase K and partial trnK gene *intron* Identities: 100%
rbcL	1 acagattgac ttattatact cccgattacg aaaccaaaga tactgatatc ttggcagcat 61 tccgagtaac tcctcaaccc ggagttcctg ctgaagaagc gggggccgcg gtagctgccg 121 aatcttctac tggtacatgg acaacggtgt ggactgatgg acttaccagt cttgatcgtt 181 acaaaggacg atgctaccac atcgaggccg ttgttgggga ggaaaatcaa tatattgctt 241 atgtagctta tcctttagac ctttttgaag aaggttctgt tactaatatg tttacttcca 301 ttgtgggtaa cgtatttggt ttcaaagccc tacgagctct acgtctggaa gatttgcgaa 361 ttcctcctgc ttattccaaa actttccaag gcccgcctca tggcatccag gttgaaagag 421 ataaattgaa caagtatggt cgtcccctat tgggatgtac tattaaacca aaattgggat 481 tatccgcaaa aaactacggt agagcggttt atgaatgtct acgtggtggg cttgatttta 541 ccaaggatga tgaaaacgtg aactcacaac cttttatgcg ttggagagac cgtttcttat 601 tttgcgctga agcaatttat aaagcgcaag ccgaaacagg tgaaatcaaa ggacattact 661 tgaatgcaac tgcgggtaca tgtgaagaaa tgatgaaaag ggctatattt gccagagaat (896 bp)	GenBank Sequence ID: MZ054605.1	MT806741.1 *Iris planifolia* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene Identities: 99.44%
trnL-trnF	1 ctgattaatt gagctttagt atggaaacct gctaagtggt aacttccaaa ttcagagaaa 61 ccctggaaaa aaaggggca atcctgagcc aaatctttat tttgagaaaa cgaacacggg 121 tttaaaaact agaataaaaa aagataggtg cagagactca atggaagctg ttctaatcaa 181 acgattaatc acgacctgaa tccattatca ttatatatgc aaaattcaga gctattgtgg 241 atctattcca atcaaagttg aaggaagaat agatcagtga tcaaatcatt cattccagag 301 tttgatagat cttttgaaaa acgaattaat cagaagagaa taaagagaga gtcccattct 361 acatgtcaat accgacaaca atgaaattta tagtaaaagg aaaatccgtc gactttagaa 421 atcgtgaggg ttcaagtccc tctatcccca ataaaaagtc catttgattt cctaactatt 481 tatattcttt ttttcatctt catccatcga tggttcaaac aaaattcaat atctttctca 541 ttaattctac tcttttcttt cacaaaagga tccaaactaa aatctttgga tcttatccca 601 atctggtttg gatagatatg atacctgtac aaatgaacat gtatgggcaa gtaattccca 661 ttattgaatc attcacaatc catatcatta tccttacgtt tacaaagaaa gtcttctttt (877 bp)	GenBank Sequence ID: MZ054615	KC510940.1 *Iris warleyensis* tRNA-Leu (trnL) gene and trnL-trnF intergenic spacer, partial sequence Identities: 99.72%

Table 6. Variability in Guanine ~ Cytosine (GC) content calculated from the target fragments

Species	*ITS*		*matK*		*rbcL*		*trnL-F*	
	length, bp	G~C %	length, bp	G~C %	length, bp	G~C %	length, bp	G~C %
I. orchioides	754	64.5	779	32.2	901	43	863	34.5
I. hippolyti	758	63.9	761	31.8	896	43	877	33.9
I. svetlanae	758	63.3	879	31.7	931	43	850	34.1
I. tubergeniana	756	65.1	776	32.6	927	42.9	861	34.5
I. sogdiana	441	65.5	915	32	526	43.7	830	34
I. vicaria			773	31.6	943	43.1		
I. warleyensis			770	31.7	845	43.1	865	34.1
I. korolkowii			773	31.3	906	43.2	860	33.8
I. magnifica			778	31.9	900	42.8	851	34
I. victoris			772	32	876	43.2	866	34.3

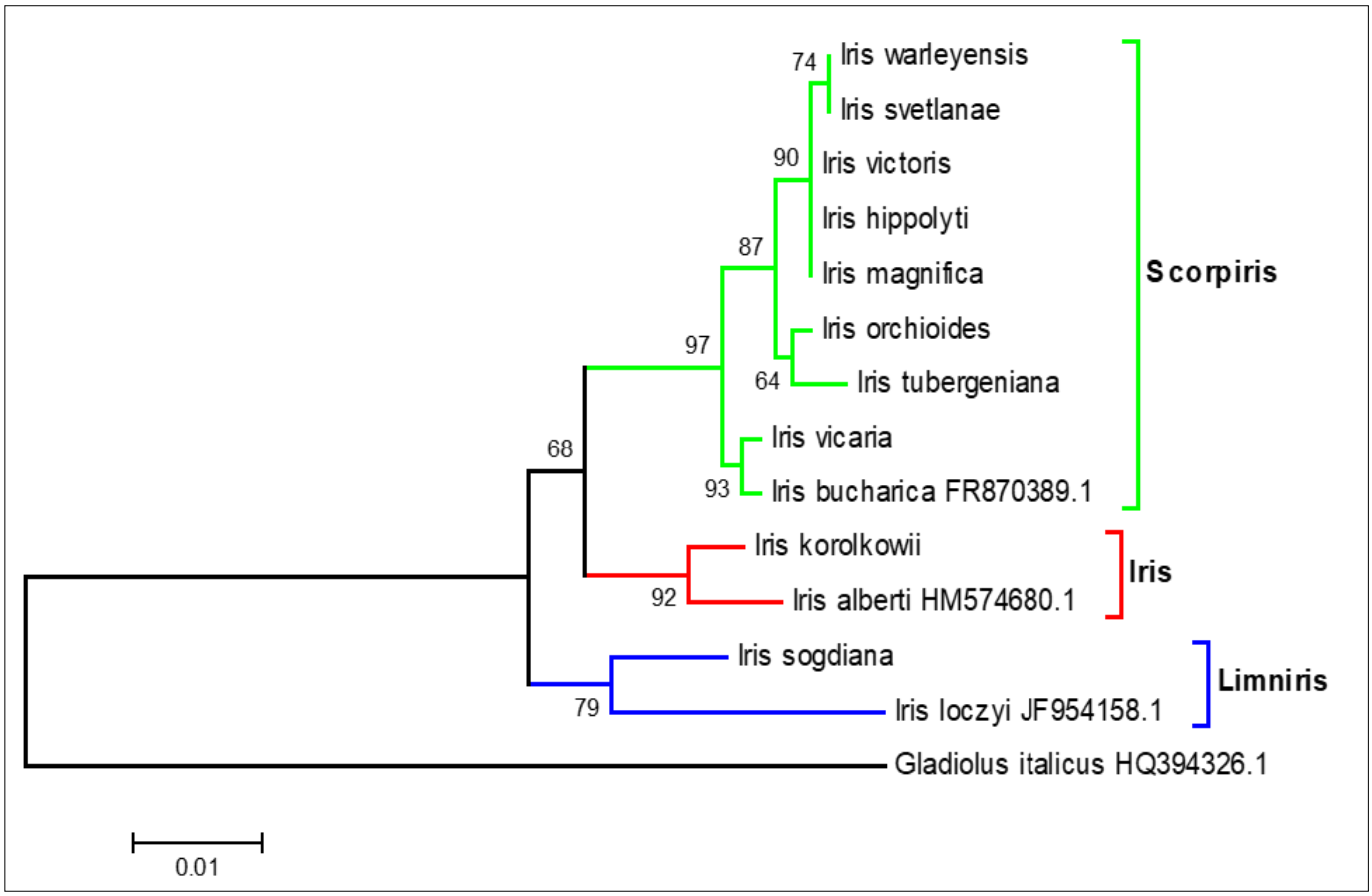

Fig.2. Hierarchical clustering of *Iris, Scorpiris, Limniris* subgenera within *Iris* genus, constructed according to the sequence comparison of the *matK* region

I. hippolyti, I. svetlanae are considered to be morphologically close species with winged claws (37, 42). Our dendrogram demonstrates the monophyly of the subgenus *Scorpiris* (38).

According Ikinci, *I. magnifica* morphologically isolated species with a well-developed stem, markedly swollen, pencil-like roots, flowers with wide wings and an entire raised crest (39).

By Wilson, *I. korolkowii* is the type species for the section *Regelia* (34), *I. alberti* is represent the section *Iris* (10, 11). They are placed together into a separately branch and forming the second subclade of the subgenus *Iris,* with Bootstrap value- 92. Our study resolved, *I. loczyi* and *I. sogdiana* are branched separately from other species, with Bootstrap value- 79, and grouped into a distinct clade forming subgenus *Limniris*. This fact is consistent with previous taxonomy (11, 39). By author (39), *I. loczyi* has simple crest and it is link between subgenus *Limniris* and other subgenera. According Mathew, subgenus *Limniris* is a beardless and rhizomatous irises with lack sepal crests (37).

The samples accessed from NCBI *I. bucharica, I. alberti, I. loczyi* are grouped appropriately, according to their classification within the genus *Iris* (11, 34, 41).

Conclusion

Thus, the successful species-level molecular identification of the genus *Iris* endemics growing on the territory of Uzbekistan has been carried out. The results of genotyping based on *ITS, matK, rbcL, trnL-F* consensus sequences for *Iris hippolyti* showed a satisfied reproducibility of amplification for all markers. Using a combinations of DNA markers, 39 consensus nucleotide sequences were obtained for 10 taxa. Our hierarchical clustering results based on *matK* sequencing for 10 representatives of the genus *Iris* are consistent with its classical morphological classification. The subgeneric position in our phylogenetic analysis is congruent with the previous molecular phylogenetic studies. So, the efficacy of utilizing DNA technology as a tool for evaluating the diversity of plant species is demonstrated in this study.

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Authors contributions

EN carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript, EO collected materials for this study, NB collaborated in the writing and editing of the manuscript, KhK participated in study design. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The author declares that the provided information has no conflict of interest

Ethical issues: None.

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