ECOLOGIA BALKANICA

2023, Vol. 15, Issue 2 December 2023 pp. 112-125

Molecular and anatomical study of alien species Sisyrinchium rosulatum (Asparagales, Iridaceae) in Bulgaria

Tsvetanka Raycheva[1], Kiril Stoyanov[1*], Elena Apostolova-Kuzova[2], Samir Naimov[2], Yulian Marinov[3]

[1] - Agricultural University – Plovdiv, Faculty of Agronomy, Department of Botany and Agrometeorology, 12 Mendeleev Blvd, Plovdiv, BULGARIA

[2] - University of Plovdiv, Faculty of Biology, Department of Plant Physiology and Molecular Biology, 24 Tzar Assen Str., Plovdiv, BULGARIA

[3] - Natural History Museum – Plovdiv, 34 Hristo G. Danov Str., Plovdiv, BULGARIA

*Corresponding author: nomtax@gmail.com

Abstract. The presented study describes some morphological and anatomical features of collected specimens of *Sisyrinchium rosulatum* E.P.Bicknell, from the first reported locality in Bulgaria. The isolated DNA sequences from the nuclear ITS1 - 5.8S rDNA - ITS2 region and the chloroplast rpoC1 gene proved the recognition of *S. rosulatum* and excluded the previously noticed *S. montanum* and *S. angustifolium*.

Key words: alien species, ITS1/2 region, neophyte, rpoC1 gene, phylogeny, anatomy, *Sisyrinchium*, Iridaceae.

Introduction

The genus *Sisyrinchium* L. contains more than 200 accepted species worldwide (Rudallet et al., 1986) This genus is native to North America but has spread rapidly over the past decade in various phytogeographic regions of the Northern Hemisphere. The natural distribution of *S. rosulatum* E. F. Bicknell is in the southeast states of the USA (Nicolella & Ardenghi, 2013). It is reported as naturalized in the Dominican Republic, Hawaii, India, Iran, Japan, Korea, Madagascar, New Caledonia, Puerto Rico, Spain, Tibet (Nicolella & Ardenghi, 2013), Italy (Nicolella & Ardenghi, 2013; Galasso et al., 2019, 2022), Bhutan (Gyeltshen et al., 2019) and Albania (Gjeta et al., 2020).

The first report for the genus *Sisyrinchium* from Bulgaria is from 1972, published as *S. angustifolium* Mill. (Kolev, 1972). Later, a notice for the genus *Sisyrinchium* as new for Bulgaria have been published (Gogushev, 1999). In the summarized publications on the flora of Bulgaria, only *S. montanum* Greene has been indicated for the southern part of The Valley of River Strouma (Assyov & Petrova, 2012). In field studies, examination of herbarium specimens and genome size examinations, *S. rosulatum* has been reported as a new species of Bulgarian flora (Stoyanov et al., 2021, 2023).

The taxonomy of *Sisyrinchium* is debatable, with many opposing views on species and synonymy. This genus exhibits problematic relationships between the species, with still disputable morphological characters. *Sisyrinchium angustifolium* and *S. montanum* are very similar in morphological characteristics, which follows to misrecognition of the two species,

like the misidentified Bulgarian representative sub *S. angustifolium* (Kolev, 1972). Some authors (Gjeta et al., 2020; Dudáš, 2022) accepted *S. angustifolium* as a synonym of *S. montanum* for the reason above. Both species have very different branching of the stem. The stem of *S. angustifolium* has several nodes and branches, each bearing a terminal inflorescence. For comparison, the stem of *S. montanum* is unbranched and has a single node holding a single terminal inflorescence. Furthermore, *S. micranthum* Cav., widely distributed worldwide and listed as invasive (Shin et al., 2016), is a sister species to *S. rosulatum* E. P. Bicknell.

Anatomical studies in the genus (Holm, 1908; Goldblatt et al., 1990) have provided some diagnostic characteristics. A study based on morphological and anatomical investigation has reported *S. angustifolium* as a new species for Türkiye (Eminagaoglu & Özcan, 2014).

Sisyrinchium is a complex polyploid genus, possessing species with difficult morphological delimitation and interspecific hybridization (Tacuatiá et al., 2017).

Based on molecular and morphological approaches, an infrageneric study of *Sisyrinchium* has suggested ten sections (Dellanhese–Inácio, 2016; Ávila–González et al., 2022). The molecular studies in this genus have collected a lot of referent genome and chloroplast sequences (Alves et al., 2014; Burgees et al., 2011; Chauveau et al., 2011; Dellanhese–Inácio et al., 2016, Lowo et al., 2018; Kang et al., 2020; Karst & Wilson, 2012).

Our study aims to establish the proper status of *Sisyrinchium* in Bulgaria. Given the high phenotypic and genotypic variability and polyploidization processes, we applied an anatomical survey and sequenced the ITS nuclear region and the rpoC1 chloroplast gene.

Materials and Methods

Evaluated specimens

The collected specimens were determined using the existing identification keys in the literature (Ingram, 1980; Fedchenko, 1935; Cholewa & Henderson, 2022; "WFO", 2023).

The evaluated specimens are listed below by floristic region (subregion in brackets), MGRS (10×10 km) square, locality description, decimal geographic coordinates (if available), altitude, date (collectors in brackets), herbarium acronym (according to "Index Herbariorum", 2023), and specimen numbers. A source for the comparative images of specimens from GJO, MO, NYBG and USF was the Global Biodiversity Information Facility ("Manual de plantas de Costa Rica", 2023; Ramirez et al., 2022; University of North Carolina at Chapel Hill Herbarium, 2023; Franck & Bornhorst, 2023; "Steiermärkisches Landesmuseum Joanneum – Herbarium GJO", 2023).

***Sisyrinchium rosulatum* E.P.Bicknell:**

BULGARIA: Valley of River Strouma (Southern): 34TFL78: Eleshnitsa village (Kolarovo), "Topilishte" locality ("Kodzha-orman"), wet meadow, 1970-06-12 (*coll.* I. Kolev) SOA s.n. (sub *S. angustifolium*); N41.37689 E23.11246, 270 m, 2020-06-25 (*coll.* G. Gogushev); N41.3766667 E23.1191667, 227 m, 2022-06-20 (*coll.* Y. Marinov, Ts. Raycheva, K. Stoyanov) SOA 063305, NCBI GenBank entry: ITS1/2 (OP800245), rpoC1 (OP806002); **34TFM82:** South of Oshtava village, Kresna district, in a wet meadow of the alliance *Cynosurion cristati*, N41.78142 E23.22613, 690 m, 2020-06-25 (*coll.* S. Stoyanov, V. Vladimirov, S. Bancheva) SOA 177433, 177434, 177435; **USA:** 1896-04-06 (*coll.* C. T. Mohr) MO-202780 – Syntypus; **17SPS026:** Charleston Co., 1853-05-08 (*coll.* L. R. Gibbes) NYBG 319485 – Typus; **17SQV17:** Raven Ridge Road, N35.917984 W78597903, 2022-04-08 (*coll.* B. England) NCU 677445; **17RNK89:** Hobe Sound, N27.05026 W80.14086, 2022-04-16 (*coll.* G. Braun & J. Habicht) USF 303551.

***Sisyrinchium angustifolium* Mill.:**

CHECH REPUBLIC: 33UYQ16: Vsetin, 1910-06-12 (*coll.* Jul. Machacek) SOM 14231; **MOLDOVA: 35TMM61:** Poiana, 1910-06-12 (*coll.* E. Topa) SOM 145748; **ROMANIA: 34TFT48:** Marmaros, 1951-07-15 (*coll.* A. Margittal) SOA 4683, 4684; **UNITED KINGDOM: 30NTL76:** Falmouth, 1902-07-06 (*coll.* C.H.Bisseel & E.B.Chaberlain) SOA 4685; **UKRAINE: 35TNK99:** Prut, 1938-07-14 (*coll.* A. Srodon) SOM 14232**; 35ULP04:** Jasina, 1932-07-18 (*coll.* K. Domin) SOM 94760; **35ULP57:** Werbinz Nizni, 1912-06-12 (*coll.* A. Wroblew-

sky) SOM 103347; **USA: 19TDG02:** Wrewster 1919-07-13 (*coll.* M.L.Fernald) SOA 4682.

***Sisyrinchium bermudiana* L.:**

IRELAND: 1898-06-22 (*coll.* L. R. Lloyd Praege) SOA 4678; **29UNU47:** SOM 105814; **FRANCE: 31TFN16**, Cote d'or: Recey, 1932-03-11 (*coll.* G. Desplantes) SOA 21865; **RUSSIA: 36VUL96:** Trubnikovo, 1967-06-18 (*coll.* T. Kolesnikova & N. Tzvelev) SOM 124521; **USA: 10SEH00:** Point Reyes Peninsula, 1963-03-30 (*coll.* H. K. Sharsmith) SOM 101178.

***Sisyrinchium groenlandicum* Böcher:**

DENMARK (Greenland): 22WES34: Kapisigdlit, N64.4166667 W50.2666667, 50-100 m, 1973-07-25 – 1973-08-05 (*coll.* B. Fredskild) SOM 135853.

***Sisyrinchium micranthum* Cav.:**

HONDURAS: 16PCB38: Cortés Dept. Pulapanzack, N15.25 E88.5667, 1985-04-20 (*coll.* C. Bendeck) USF 194248; **16PDA87:** Francisco Morazán Dept. Cerro Cantagallo, 1780 m, N14.25 W87.1667, 1984-02-11 (*coll.* J. M. Berríos) USF 195698.

***Sisyrinchium montanum* Greene:**

AUSTRIA: 33TVN98: Seehöhe, 550 m, N47.68556 E14.92389, 2020-08-07 (*coll.* K. Zernig) GJO 0103737; **33TUN95:** Seehöhe, 1130 m, N47.41806 E13.65194, 2019-06-19 (*coll.* I. Wendelin) GJO 0097169; **UKRAINE: 35UKP92:** Kvask, 1200 m, 2006-07-28 (*coll.* V. Goncharenko & A. Tashev) SOM 163726.

***Sisyrinchium mucronatum* Michx.:**

USA: 1979-07-19 SOM 139965; **17TNE78:** Sewickley, 1979-07-19 SOM 138965; **18TXM71:** Plainville, 1905-05-27 – 1905-07-23 (*coll.* C. H. Bissell) SOA 4687.

***Sisyrinchium tenuifolium* Humb. & Bonpl.:**

MEXICO: 1885-08-30 SOA 23595; 1885-08-30 SOM 14230.

Anatomical observations

Ten plants from the evaluated locality (SOA 063305) were conserved in 75% alcohol. For anatomical observation, the transverse sections of the stem and leaf (middle and sheath parts) were taken by hand using a razor. The epidermis was peeled from the leaves by hand with tweezers. The plant material was mounted without staining on temporary slides using glycerol. The microscopic slides were observed and photographed using a Motic Panthera digital microscope. The anatomical characters were measured using Micam 2.4 software (Van Westen, 2019).

DNA extraction, amplification, and sequencing

The plant genomic DNA was extracted and purified using Dneasy Plant Mini Kit (QIAGEN, Germany), as was described earlier (Apostolova et al., 2022).

The quality of the resulting DNA was assessed spectrophotometrically, using Epoch™ Microplate Spectrophotometer (USA), and DNA integrity was evaluated by 1% agarose gel electrophoresis.

The DNA fragment encoding for ITS1 – 5.8S rDNA – ITS2 cluster was amplified using the following primers: ITS–A (5′–GGAAGGAGAAGTCGTAACAAGG–3′) and ITS–B (5′–CTTTTCCTCCGCTTATTGATATG–3′) (Tirel et al., 1996), as described earlier (Raycheva et al., 2022).

The RNA polymerase subunit beta encoding chloroplast gene (rpoC1) was amplified using the following primers: rpoC1 F (5′–GTGGATACACTTCTTGATAATGG–3′), and rpoC1 R5′–TGAGAAAACATAAGTAAACGGGC–3′) described by Anvarkhah et al. (2013).

The reactions were set in a final volume of 50 µL containing 1x reaction buffer, 200 µM of dNTPs, 0.2 µM of each primer, 100 ng of genomic DNA, and one unit of Q5 High Fidelity DNA polymerase (New England Biolabs). The PCR amplification was conducted under the following parameters: initial denaturation at 94°C for 45 s, followed by 30 cycles at 94°C for 10 s for denaturation, 10 s at 62°C for primer annealing, 30 s at 72°C for primer extension, and a final elongation step of 2 min at 72°C. Amplified PCR products were separated by 0.8% agarose gel electrophoresis, excised from the gel, and purified using a QIAquick Gel Extraction Kit (QIAGEN, Germany). The purified DNA fragments were bidirectionally sequenced in a Eurofins facility (Eurofins Genomics, Germany).

The obtained DNA sequences from the amplified target nuclear and chloroplast regions were deposited in the NCBI database. They are available in the database under

accession numbers OP800245 and OP806002, respectively.

Phylogenetic analysis

The obtained nucleotide sequences were compared against those already deposited and available in the NCBI Nucleotide database. The DNA sequences included in the phylogenetic analysis were selected based on the coefficients obtained in the BLAST analysis. The best hits (Tables 1, 2) were downloaded and included in phylogenetic analysis following the previously published models (Dellanhese–Inácio et al., 2016). Some species of the outstanding sections were used to form the outgroup. The alignment of the sequences was achieved using the ClustalW Multiple alignments (Thompson et al., 1994). The results (Figs 6, 7) were visualized using CLC Sequence Viewer 8.0. The phylogenetic analysis of the ITS sequences was conducted using Bayesian phylogenetic inference with MrBayes 3.2 (Ronquist et al., 2012) using 2×4 chains for two million generations, nuclear data set ГТР + G + I, sampling tree per 1000 generations, two independent runs. The result was visualized as a phylogenetic tree using TreeGraph 2 (Stöver & Müller, 2010).

Table 1. Compared sequences of ITS1/2 (18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence) data taken from NCBI GenBank database

Species	Origin	GenBank entry number	Publication
S. rosulatum	USA	JN389254	Karst & Wilson, 2012
S. rosulatum	USA	JN389217	Karst & Wilson, 2012
S. rosulatum	Georgia	JN389263	Karst & Wilson, 2012
S. rosulatum		JN389277	Karst & Wilson, 2012
S. micranthum	Argentina	JN389261	Karst & Wilson, 2012
S. micranthum	Argentina	JN389260	Karst & Wilson, 2012
S. micranthum	Peru	JN389213	Karst & Wilson, 2012
S. micranthum	Colombia	JN389262	Karst & Wilson, 2012
S. angustifolium		HQ607040	Chauveau et al., 2011
S. angustifolium	Canada	JN389238	Karst & Wilson, 2012
S. demissum	Mexico	JN389284	Karst & Wilson, 2012
S. idahoense	USA	JN389246	Karst & Wilson, 2012
S. macranthum	Argentina	JN389257	Karst & Wilson, 2012
S. montanum	USA	JN389249	Karst & Wilson, 2012
S. minutiflorum	Venezuela	JN389259	Karst & Wilson, 2012
S. praealtum	Peru	JN389275	Karst & Wilson, 2012
S. scabrum	Mexico	JN389278	Karst & Wilson, 2012
S. scabrum	Mexico	JN389279	Karst & Wilson, 2012
S. tenuifolium	Mexico	JN389282	Karst & Wilson, 2012
S. tinctorium	Brazil	JN389286	Karst & Wilson, 2012
S. tinctorium	Peru	JN389283	Karst & Wilson, 2012
S. tinctorium	USA	JN389256	Karst & Wilson, 2012
S. xerophyllum	USA	JN389285	Karst & Wilson, 2012
S. uliginosum		HQ607102	Chauveau et al., 2011
S. uliginosum		KF577152	Alves et al., 2014
S. megapotamicum		HQ607061	Chauveau et al., 2011
S. californicum	USA	JN389241	Karst & Wilson, 2012

Table 2. Compared sequences of SP085 RNA polymerase beta' subunit (rpoC1) gene, partial cds; chloroplast data are taken from NCBI GenBank database

Species	GenBank entry number	Publication
S. rosulatum	HQ606527	Chauveau et al., 2011
S. micranthum	MH177071	Lowo et al., 2018
S. micranthum	KX432356	Dellanhese–Inácio et al., 2016
S. angustifolium	KX432311	Dellanhese–Inácio et al., 2016
S. angustifolium	HQ606491	Alves et al., 2014
S. angustifolium	NC_056184	Kang et al., 2020
S. montanum	HQ594135	Burgees et al., 2011
S. montanum	HQ606521	Chauveau et al., 2011

Results

Anatomical features of stem and leaf based on Bulgarian materials

The stem (Fig. 1) has wings, making it 1.1–1.8 mm wide. The cortex in the wing zone contains several layers of chlorenhymatous cells. The core of the stem, along with the sclerenchymatous hypodermis, has a section of 500–760 × 400–560 µm. The hypodermis' thickness ranges from 21 to 98 µm. The collateral vascular bundles are 16–20 on a count, arranged in 1–2 circles, with parenchymatous endodermis. The size of the vascular bundles in the core is 17–66 × 23–44 µm. Each wing has 1–2 vascular bundles, surrounded by endodermis and without sclerenchyma. The epidermal cells in this zone are thickened and protruding, with a thick cuticle. The stomata are rarely observed, sunk in deep crypts 8.7–24.1 µm below the level of the epidermis.

The leaves are isolateral, 66–238 µm thick (Figs 2, 3). The count of the vascular bundles is 10–18 in cross–section. The vascular bundles in cross–section are 21–121 µm thick and 18–82 µm wide, surrounded by endodermis. The widest vascular bundle continues from the keel of the wing and has a sclerenchymatous zone, generally directed to the sheath, rarely taking up to half of the vascular bundle. The chlorenchyma is uniform, consisting of homogeneous, rounded cells 13–31 × 11–39 µm, with few chloroplasts in the medular area. The epidermis of the leaf blade is 17–28 µm thick. The epidermal cells are 81–1072 µm long and 14–37 µm wide, uniformly wide in the area over the vascular bundles and swelling in the stomatal area (Fig. 3). Papillae are not present. The adaxial cell walls of the epidermal cells thickened, covered by a thin smooth cuticular layer. The stomata are anomocytic, situated on both leaf surfaces, and arranged in parallel rows (Fig. 4, 5). The stomatal area is elliptic, 13–35 × 8–19 µm. The guard cells in a cross–section are 4–10 µm thick and 6–12 µm wide. The stomata are hidden in crypts 9–24 µm deep. The sheath epidermis is uniform, 19–22 µm thick, without stomata.

All measured characteristics are listed in Table 3.

Fig. 1. Stem cross–section. Scale bar: 200 µm.

Fig. 2. Leaf cross–section in the zone of the sheath. Scale bar: 200 µm.

Fig. 3. Leaf cross–section in the zone of the blade. Scale bar: 200 µm.

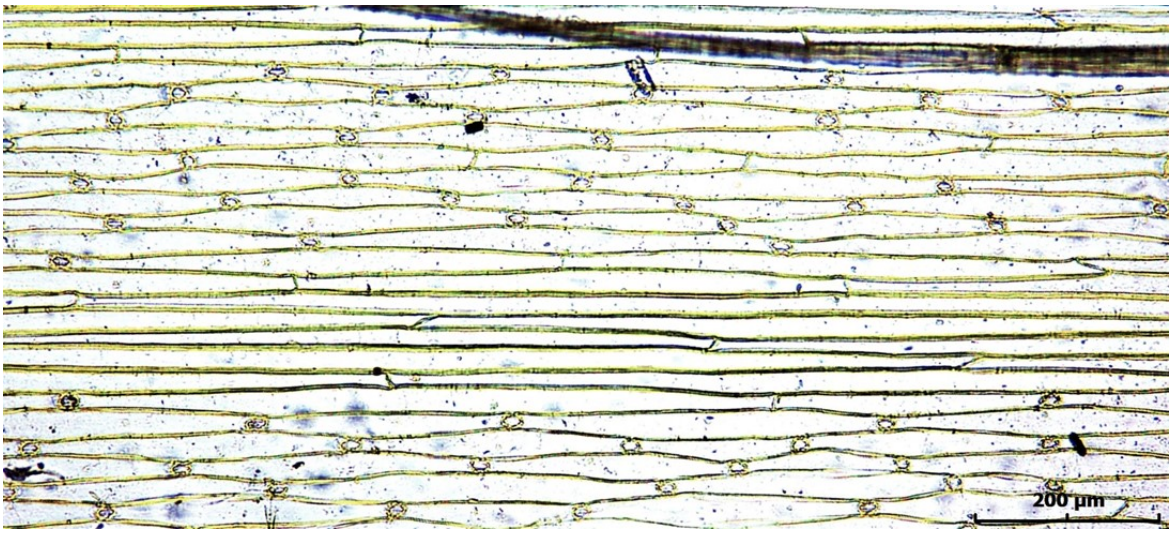

Fig. 4. Leaf epidermis of *Sisyrinchium rosulatum* (scale bar: 200 µm)

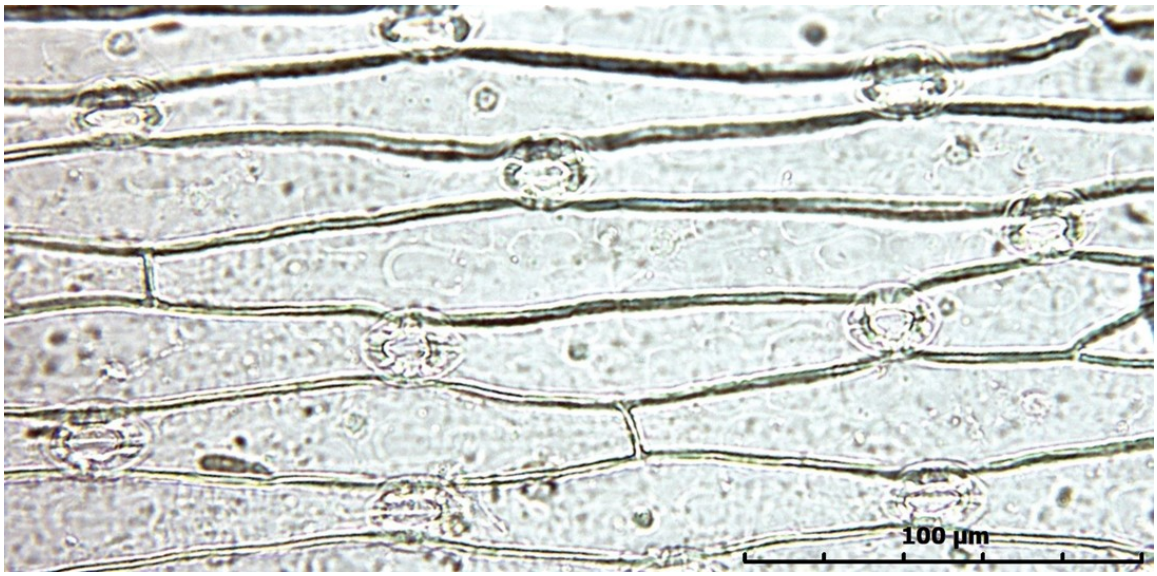

Fig. 5. Stomata on leaf epidermis of *Sisyrinchium rosulatum* (scale bar: 100 µm).

Table 3. Metric anatomical and morphological features of *Sisyrinchium rosulatum* in Bulgaria

Measured feature	min–max	Mean ± standard deviation
Capsule and seed		
Capsule length, mm	2.79–5.71	4.05 ± 0.81
Capsule width, mm	2.49–4.09	3.33 ± 0.4
Seed length, mm	0.73–1	0.9 ± 0.82
Seed width, mm	0.62–0.92	0.75 ± 0.1
Stem		
Width (together with the wing), µm	1132–1837	1538 ± 327.1
Thickness, µm	403–689	607.14 ± 109.76
Core, width, µm	500–760	672.86 ± 110.8
Core, thickness, µm	400–558	499.57 ± 68.29
Sclerenchyma ring thickness, µm	21.3–98	51.13 ± 15.67
Epidermis – thickness, µm (stem)	12–35.6	22.01 ± 4.53
Vascular bundles – count (stem)	10–18	13.67 ± 3.57
Vascular bundle – a, µm (stem)	17.2–66	13.67 ± 3.57
Vascular bundle – b, µm (stem)	22.9–43.8	33.38 ± 5.79
Leaf		
Width, mm	0.9–4.1	2.29 ± 0.76
Thickness, µm	66–238	156.42 ± 37.16
Epidermis - thickness, µm (leaf)	10–34.2	23.88 ± 4.49
Vascular bundle – a, µm (leaf)	21.4–121	62.44 ± 25.52
Vascular bundle – b, µm (leaf)	17.8–82	48.6 ± 17.39
Epidermal cells, length, µm	81–1072	295.08 ± 189.41
Epidermal cells, width, µm	13.9–36.6	23.76 ± 5.17
Stomatal area length, µm	13.3–34.8	22.99 ± 4.13
Stomatal area width, µm	7.8–19.4	13.46 ± 2.3
Epidermal cells thickness, µm	16.8–37.4	26.44 ± 4.07
Epidermal cells cuticle, µm	2.3–10.1	5.76 ± 1.66
Epidermal cells width, µm	11.8–35.3	21.04 ± 4.77
Guard cells height, µm	3.5–9.7	6.75 ± 1.5
Guard cells width, µm	6–12	8.73 ± 1.26
Stomatal crypt, µm	8.7–24.1	16.17 ± 3.25
Parenchymatous cells height, µm	13.5–31.1	22.05 ± 4.77
Parenchymatous cells width, µm	11.2–38.9	20.31 ± 5.44
Sheath epidermal cells thickness, µm	10.2–21.8	16.59 ± 3.7
Sheath epidermal cells width, µm	18.7–29.8	24 ± 4

DNA sequences

The BLAST analysis of the rDNA ITS1/2 nuclear region showed the highest similarity of the investigated specimen (OP800245) with *S. rosulatum* and *S. micranthum* (Table 2, Fig. 6). On the other hand, the BLAST analysis of the rpoC1 gene (OP806002) showed that it is too conservative for the study's aims (Table 3, Fig. 7) but also displayed a high similarity with *S. rosulatum.*

In the phylogenetic tree based on the ITS1/2 region (Fig. 8), the Bulgarian specimen OP800245 is placed in a branch with the rest of the members of section *Morphanthus* and outstayed away section *Sisyrinchium*, with the species wrongly reported for Bulgaria being *S. montanum* and *S. angustifolium*. The members of *Morphanthus* (*S. rosulatum* and *S. micranthum*) remain unconstrained. More conservative is the result of the alignment of the rpoC1 chloroplast gene. The evaluated sequences are almost identical, showing only two substituted bases at positions 188 and 349 (Fig. 7).

Fig. 6. Alignment of the evaluated ITS1/2 sequences (part 1).

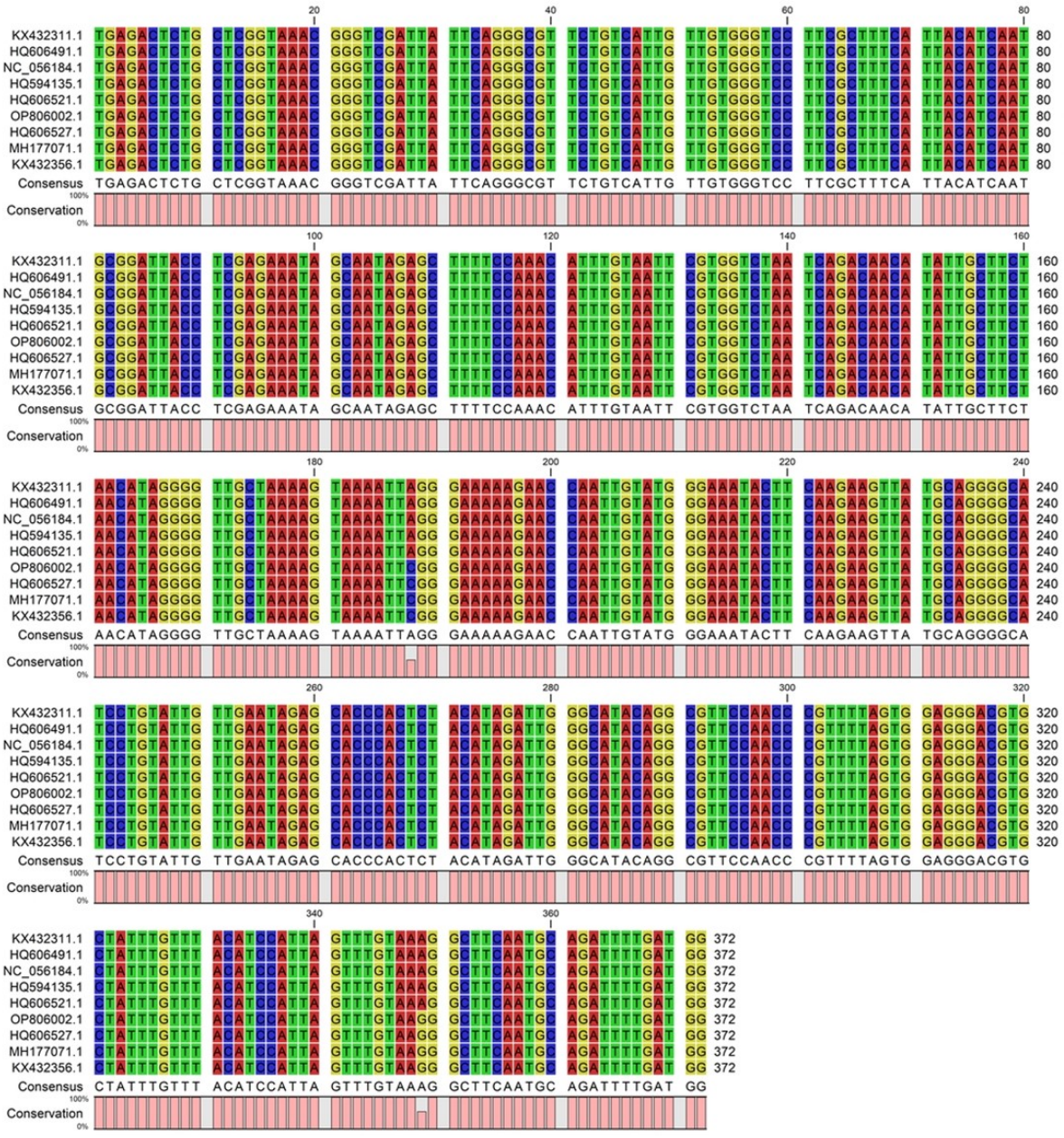

Fig. 7. Alignment of the evaluated rpoC1 sequences

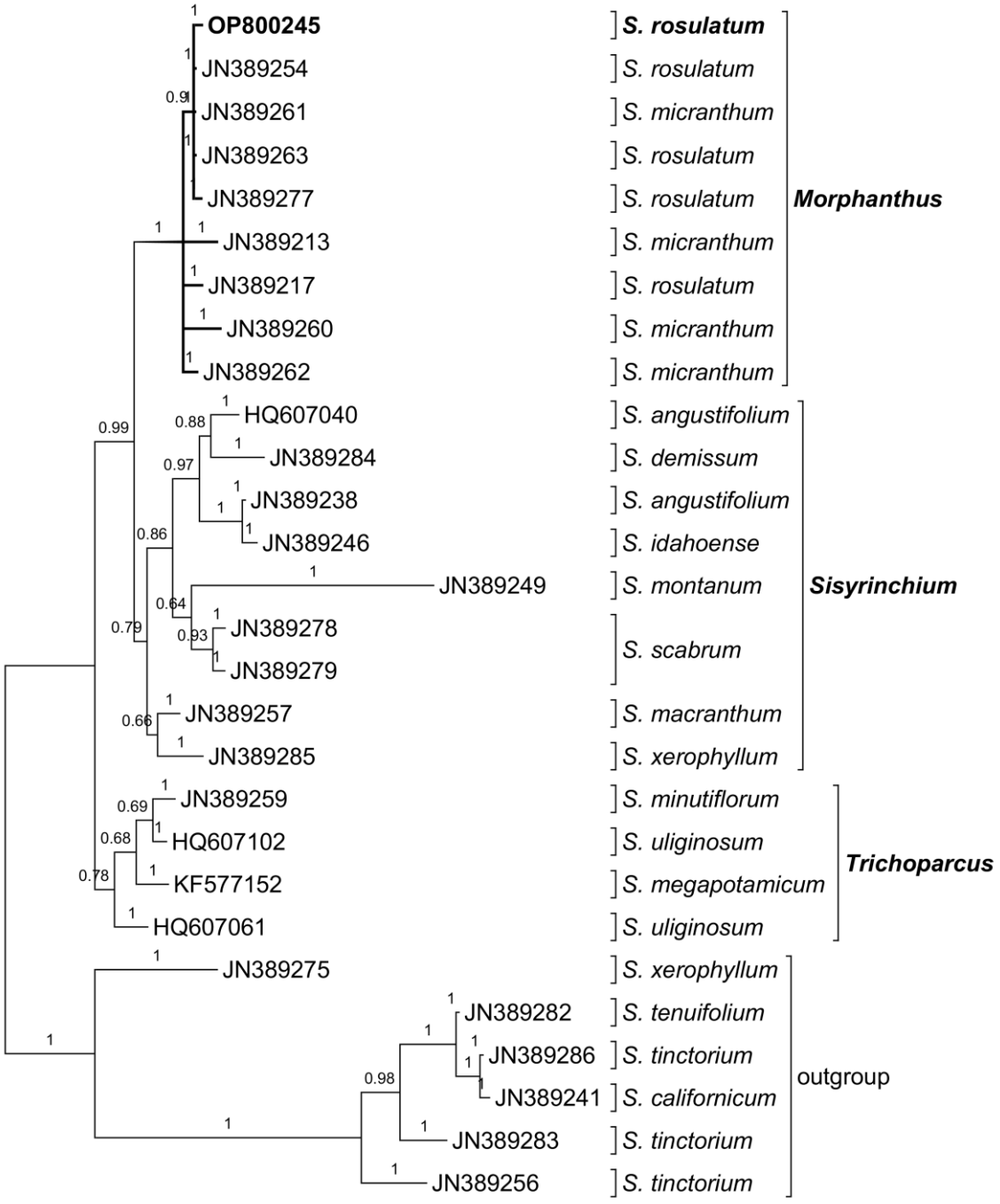

Fig. 8. Phylogenetic relationships between *Sisyrinchium* isolates based on partial ITS1/2 region.

Discussion

The anatomy of *S. rosulatum* is consistent with the results of previous studies. The chlorenchyma structure in sections *Bermudiana* and *Eriphilema* is of more or less palisade–like differentiated cells (Holm, 1908; Goldblatt et al., 1990), whereas in *Echthronema*, this tissue consists of uniform elongated cells. That study has not included *S. rosulatum* but provided data for *S. montanum* and *S. angustifolium*. These species have a palisade structure of the chlorenchyma, in contrast to the uniform tissue observed in *S. rosulatum*. A study has reported *S. angustifolium* as a new species for Türkiye (Eminagaoglu & Özcan, 2014). The published anatomical features have been similar to our observations in *S. rosulatum*, e.g. the lack of palisade parenchyma. This fact implies uncertainty in the determination of the materials from Türkiye. Also, the authors reported a new tetraploid chromosome number for *S. angustifolium*,

which remains unconfirmed by other research. There are no NCBI–deposited DNA sequences of *Sisyrinchium* species from Türkish localities. The diagnostic description of *Sisyrinchium* specimens in Türkiye (Eminagaoglu & Özcan, 2014) is similar to those of *S. rosulatum* in literature data and our collected plants. Also, the conditions of habitats and altitudes are similar for Türkish and Bulgarian collections. The explanation above suggests that *S. rosulatum* might have a broader distribution in the Balkans.

The explained contradictory and uncertain taxonomic treatments of the *Sisyrinchium* representatives point to the need for additional research on Balkan populations. The participation of *S. montanum* in the Bulgarian flora could be possible. Until now, it has remained unnoticed and unconfirmed in the existing collections. The closest known location for *S. montanum* has been discovered on the coast of Vlasinsko Lake in Eastern Serbia (Ranđelović & Zlatković, 2010), near the Bulgarian border. The spread of S. *montanum* is slow in Europe, favouring anthropogenically disturbed sites. Its low competitiveness with local species in the Czech Republic is a reason to be qualified as a naturalized neophyte (Pyšek et al., 2012).

Our phylogenetic tree (Fig. 8) was based on 28 sequences of the ITS region from 18 species (Fig. 6) and agreed with the previously proposed phylogeny within the genus (Dellanhese–Inácio et al., 2016; Ávila–González et al., 2022). The ITS region was insufficient as a marker to delimit the species but clearly distinguished the known sections. The tree consisted of the closely related taxa from the sections *Morphanthus*, *Sisyrinchium*, *Trichoparcus*, and the rest formed the outgroup. *Sisyrinchium rosulatum* and *S. micranthum*, together with the specimen from Bulgaria, took their place in the section *Morphanthus*. The discussed species, *S. montanum* and *S. angustifolium* remained in a different branch – sect. *Sisyrinchium*. Sect. *Trichoparcus* was the most outstanding branch of the three closely related sections.

Despite the high conservatism of the rpoC1 gene, the alignment showed two key loci (Fig. 7) sufficient to demonstrate that the Bulgarian representative of the genus *Sisyrinchium* belongs to the section *Morphanthus*. These loci delimited the section *Morphanthus*, represented by *S. rosulatum* and *S. micranthum*, of the section *Sisyrinchium*, represented by the two species wrongly reported for Bulgaria, *S. angustifolium* and *S. montanum*. At the 188th position in the alignment (Fig. 7), the rpoC1 sequences of section *Morphanthus* have nucleotide C and those of section *Sisyrinchium* with nucleotide A. In the 349th position, the section *Morphanthus* has the nucleotide G, while the section *Sisyrinchium* has A on the same locus.

The noticed point mutations, stabilized after a long evolution in the working rpoC1 chloroplast gene, look like a suitable molecular feature for recognition inside the genus. The explained couple of nucleotide swaps is enough for our aim to prove the proper species in the area of the Balkan Peninsula. The difference in the ITS sequences is a result of the neutral evolution. Based on these two opposite DNA markers, we could conclude the presence of *S. rosulatum* in Bulgaria.

In brief, the two opposite molecular markers, combined with the morphological and anatomical metrics, proved that the Bulgarian materials belong to the section *Morphanthus*. *S. rosulatum* can be distinguished from the closely related *S. micranthum* by some morphological features – longer and broader leaf blades, acuminate but not aristate tepals, and a bit bigger capsules.

Conclusions

Both the anatomical and molecular data confirmed the presence of *Sisyrinchium rosulatum* in Bulgarian flora and excluded the morphologically similar taxa. For the first time, sequences from *S. rosulatum* have been isolated and annotated from specimens from the Balkan Peninsula. They could be used in phylogenetic studies of the species in the Palearctic realm. The expanding distribution of *Sisyrinchium* species in various phytogeographic regions outside the Nearctic realm suggests a character of boreal cosmopolitans. The findings of this study could help improve identification in future *Sisyrinchium* research and help resolve some taxonomic and phylogenetic questions concerning critical and controversial taxa in the genus.

Acknowledgements

This research was funded by Bulgarian National Science Fund, grant number KP-06-N31/5.

References

Alves, T.L.S., Chauveau, O., Eggers, L. & de Souza–Chies, T.T. (2014). Species discrimination in *Sisyrinchium* (Iridaceae): assessment of DNA barcodes in a taxonomically challenging genus. *Molecular Ecology Resources*, 14(2), 324–335. doi: 10.1111/1755-0998.12182

Anvarkhah, S., Khajeh–Hosseini, M., Rashed Mohassel, M.H., Davari Edalat Panah, A. & Hashemi, H. (2013). Identification of three species of genus *Allium* using DNA barcoding. *International Journal of Agriculture and Crop Sciences*, 5, 1195–1203.

Apostolova, E., Raycheva, T., Stoyanov, K. & Naimov, S. (2022). A study of Genetic Relationships of *Crocus* Taxa, Series *Biflori* (Iridaceae) from Bulgaria by ISSR Markers. *Comptes rendus de l'Academie bulgare des Sciences*, 75(7), 992–999. doi: 10.7546/CRABS.2022.07.07

Assyov, B. & Petrova, A. (Eds) (2012). *Conspectus of the Bulgarian Vascular Flora. Distribution Maps and Floristic Elements.* 4'th ed. Bulgarian Biodiversity Foundation: Sofia, Bulgaria, 156–157. Retrieved from URL https://archive.org/details/ConspectusOfTheBulgarianVascularFlora.FourthEdition

Ávila-González, H., González-Gallegos, JG., Munguía-Lino, G. & Castro-Castro, A. (2022). The Genus *Sisyrinchium* (Iridaceae) in Sierra Madre Occidental, Mexico: A New Species, Richness and Distribution. *Systematic Botany*, 47(2), 319–334. doi: 10.1600/036364422X16512564801641

Burgess, K.S., Fazekas, A.J., Kesanakurti, P.R., Graham, S.W., Husband, B.C., Newmaster, S.G., Percy, D.M., Hajibabaei, M. & Barrett, S.C.H. (2011). Discriminating plant species in a local temperate flora using the rbcL+matK DNA barcode. *Methods in Ecology and Evolution*, 2(4), 333–340. doi: 10.1111/j.2041-210X.2011.00092.x

Chauveau, O., Eggers, L., Raquin, C., Silveiro, A., Brown, S., Couloux, A., Cruaud, C., Kaltchuk-Santos, E., Yockteng, R., Souza–Chies, T.T. & Nadot, S. (2011). Evolution of oil-producing trichomes in *Sisyrinchium* (Iridaceae) insights from the first comprehensive phylogenetic analysis of the genus. *Annals of Botany*, 107(8), 1287–1312. doi: 10.1093/aob/mcr080

Cholewa, A. & Henderson, D. *Sisyrinchium* L. In Flora of North America; Flora of North America Association. Vol. 26. Retrieved from URL http://floranorthamerica.org/Sisyrinchium

CLC Sequence Viewer 8.0, QIAGEN. Retrieved from URL https://clc-sequence-viewer.software.informer.com/8.0/

Dellanhese-Inácio, C., Chauveau, O., Souza-Chies, T.T., Sauquet, H. & Eggers, L. (2016). An updated phylogeny and infrageneric classification of the genus *Sisyrinchium* (Iridaceae): Challenges of molecular and morphological evidence. *Taxon*, 66(6), 1317–1348. doi: 10.12705/666.4

Dudáš, M. (2022). Naturalisation of Strict blue–eyed grass *Sisyrinchium montanum* Greene (Iridaceae) in Slovakia. *BioInvasions Records*, 11(2), 312–319, doi: 10.3391/bir.2022.11.2.02

Eminagaoglu, Ö. & Özcan, M. (2014). Systematics of *Sisyrinchium angustifolium* Mill. (Iridaceae), a newly recorded species from Turkey. *Bangladesh Journal of Plant Taxonomy*, 21(2), 175–180. doi: 10.3329/bjpt.v21i2.21357

Fedchenko, B.A. (1935). *Sisyrinchium angustifolium* Mill. In Komarov VL (Ed) Flora U.R.S.S. Editio Academiae Scientiarum URSS: Leningrad, Vol. 4, 577–578. (in Russian)

Franck, A.R. & Bornhorst, K. (2023). University of South Florida Herbarium (USF). Version 7.377. USF Water Institute. Occurrence dataset. doi: 10.15468/mdnmzb (accessed via GBIF.org 31.01.2023)

Galasso, G., Domina, G., Andreatta, S., Angiolini, C., Ardenghi, N.M.G., Aristarchi, C., Arnoul, M., Azzella, M.M., Bacchetta, G., Bartolucci, F., Bodino, S., Bommartini, G., Bonari, G., Buono, S., Buono, V., Caldarella, O., Calvia, G., Corti, E., D'Antraccoli, M., De Luca, R., De Mattia, F., Di Natale, S., Di Turi, A., Esposito, A., Ferretti, G., Fiaschi, T., Fogu, M.C., Forte, L., Frigerio, J., Gubellini, L., Guzzetti, L., Hofmann, N., Laface, V.L.A., Laghetti, G., Lallai, A., La Rosa, A., Lazzaro, L., Lodetti, S., Lonati, M., Luchino, F., Magrini, S., Mainetti, A., Marignani, M., Maruca, G., Medagli, P., Mei, G., Menini, F., Mezzasalma, V., Misuri, A., Mossini, S., Mugnai, M., Musarella, C.M., Nota, G., Olivieri, N., Padula, A., Pascale, M., Pasquini, F., Peruzzi, L., Picella, G., Pinzani, L., Pirani, S., Pittarello, M., Podda, L., Enri, S.R., Rifici, C.D., Roma-Marzio, F., Romano, R., Rosati, L., Scafidi, F., Scarici, E., Scarici, M., Spampinato, G., Stinca, A., Wagensommer, R.P., Zanoni, G.

& Nepi, C. (2019). Notulae to the Italian alien vascular flora: 8. *Italian Botanist*, 8, 63-93. doi: 10.3897/italianbotanist.8.48621

Galasso, G., Domina, G., Andreatta, S., Argenti, C., Astuti, G., Bacaro, G., Bacchetta, G., Bagella, S., Banfi, E., Barberis, D., Bartolucci, F., Bernardo, L., Bonari, G., Brundu, G., Buccomino, G., Calvia, G., Cancellieri, L., Capuano, A., Celesti-Grapow, L., Conti, F., Cuena-Lombraña, A., D'Amico, F.S., De Fine, G., de Simone, L., Del Guacchio, E., Emili, F., Fanfarillo, E., Fascetti, S., Fiaschi, T., Fois, M., Fortini, P., Gentili, R., Giardini, M., Hussain, A.N., Iamonico, D., Laface, V.L.A., Lallai, A., Lazzaro, L., Lecis, A.P., Ligato, E., Loi, G., Lonati, M., Lozano, V., Maccherini, S., Mainetti, A., Mascia, F., Mei, G., Menini, F., Merli, M., Montesano, A., Mugnai, M., Musarella, C.M., Nota, G., Olivieri, N., Passalacqua, N.G., Pinzani, L., Pisano, A., Pittarello, M., Podda, L., Posillipo, G., Potenza, G., Probo, M., Prosser, F., Quaglini, L.A., Ravetto Enri, S., Rivieccio, G., Roma-Marzio, F., Rosati, L., Selvaggi, A., Soldano, A., Stinca, A., Tasinazzo, S., Tassone, S., Terzi, M., Vallariello, R., Vangelisti, R., Verloove, F. & Lastrucci, L. (2022). Notulae to the Italian alien vascular flora: 14. *Italian Botanist*, 14, 99-118. doi: 10.3897/italianbotanist.14.97758

Gjeta, E., Titus, J. & Titus, P. (2020). *Galium setaceum* Lam. and *Sisyrinchium rosulatum* E. P. Bicknell, Two New Species for the Albanian Flora, Distribution and Ecology. *International Journal of Innovative Approaches in Agricultural Research*, 4(2), 269–277. doi: 10.29329/ijiaar.2020.254.11

Gogushev, G. (1999). A New genus for the Bulgarian flora. *Gora*, 5, 16–17. (in Bulgarian)

Goldblatt, P., Rudall, P. & Henrich, J.E. (1990). The genera of the *Sisyrinchium alliance* (Iridaceae–Iridoideae): phylogeny and relationships. *Systematic Botany*, 15, 497-510. doi: 10.2307/2419365

Gyeltshen, P., Borah, D. & Das, A.P. (2019). *Sisyrinchium rosulatum* E. P. Bicknell (Iridaceae): new record of a naturalized species in Bhutan. *Pleione*, 13(1), 188–191. doi: 10.26679/Pleione.13.1.2019.188-191

Holm, T. (1908). *Sisyrinchium*: Anatomical Studies of North American Species. *Botanical Gazette*, 46(3), 179–192.

Index Herbariorum, NYBG, Retrieved from URL https://sweetgum.nybg.org/science/ih/

Ingram, R. (1980). *Sisyrinchium* L. In: Tutin, T.G., Heywood, V.H., Burges, N.A., Moore, D.M., Valentine, D.H., Walters, S.M. & Webb. D.A. (Eds) *Flora Europaea* Vol. 5, Cambridge University Press: London, UK. 86–87.

Kang, Y.J., Kim, S., Lee, J., Won, H., Nam, G.H. & Kwak, M. (2020). Identification of plastid genomic regions inferring species identity from de novo plastid genome assembly of 14 Korean–native *Iris* species (Iridaceae). *PLoS ONE*, 15(10), e0241178. doi: 10.1371/journal.pone.0241178

Karst, L. & Wilson, C.A. (2012). Phylogeny of the New World genus *Sisyrinchium* (Iridaceae) based on analyses of plastid and nuclear DNA sequence data. *Systematic Botany*, 37(1), 87–95. doi: 10.1600/036364412X616666

Kolev, I.D. (1972). *Sisyrinchium angustifolium* Mill.: a new species to Bulgarian flora. *Rastenievudni Nauki*, 9(5), 177–179. (in Bulgarian)

Lowo, J., Winkworth, R.C., Gil, A.S.B., Amaral, M.C.E, Bittrich, V. & Mello-Silva, R. (2018). A revised genus–level taxonomy for Trimezieae (Iridaceae) based on expanded molecular and morphological analyses. *Taxon*, 67(3), 503–520. doi: 10.12705/673.4

Manual de plantas de Costa Rica Retrieved from URL http://legacy.tropicos.org/Name/16600570?tab=images&projectid=66

NCBI GenBank Nucleotide Database. Retrieved from URL https://www.ncbi.nlm.nih.gov/genbank/

NCBI. Standard Nucleotide BLAST. Retrieved from URL https://blast.ncbi.nlm.nih.gov/Blast.cgi

Nicolella, G., Ardenghi, N.M.G. (2013). *Sisyrinchium rosulatum* E.P.Bicknell (Iridaceae) alloctona nuova per l'Italia. *Acta Plantarum*, Notes 2, 102–106. Retieved from URL https://www.researchgate.net/publication/259384051_Sisyrinchium_rosulatum_EP_Bicknell_Iridaceae_alloctona_nuova_per_l'Italia

Pyšek, P., Danihelka, J., Sádlo, J., Chrtek, J.Jr., Chytrý, M., Jarošík, V., Kaplan, Z., Krahulec, F., Moravcová, L., Pergl, J., Štajerová, K. & Tichý, L. (2012). Catalogue of alien plants of the Czech Republic (2nd edition): checklist update, taxonomic diversity and invasion patterns. *Preslia*, 84, 155–255. Retrieved from URL https://www.preslia.cz/P122Pysek.pdf

Ramirez, J., Watson, K., McMillin, L., Gjieli, E. & Sessa, E. (2022). The New York Botanical Garden Herbarium (NY). Version 1.56. 2022, The New York Botanical Garden. Occurrence dataset. Retrieved from URL https://doi.org/10.15468/6e8nje

Randđelović, V. & Zlatković, B. (2010). Flora i vegetacija Vlasinske visoravni. Prirodno–matematički fakultet, Niš 2010. P. 113.

Raycheva, T., Stoyanov, K., Naimov, S., Apostolova-Kuzova, E. & Marinov, Y. (2022). *Crocus pallidus* (Iridaceae) – A Neglected Species for the Bulgarian Flora and Critical Taxon in the Balkans. *Plants*, 11, 686. doi: 10.3390/plants11050686

Ronquist, F., Teslenko, M., Van der Mark, P., Ayres, D.L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P. (2012). MrBayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 61, 539–542. doi: 10.1093/sysbio/sys029

Rudall, P., Kenton, A.Y. & Lawrence, T.J. (1986). An anatomical and chromosomal investigation of *Sisyrinchium* and allied genera. *Botanical Gazette*, 147(4), 466-477. Retrieved from URL https://www.jstor.org/stable/2474565

Shin, H.W., Kim, M.J., Lee, N.S. (2016). First report of a newly naturalized *Sisyrinchium micranthum* and a taxonomic revision of *Sisyrinchium rosulatum* in Korea. *Korean Journal of Plant Taxonomy*, 46(3), 295–300. doi: 10.11110/kjpt.2016.46.3.295

Steiermärkisches Landesmuseum Joanneum – Herbarium GJO. Steiermärkisches Landesmuseum Joanneum – Herbarium GJO. Occurrence dataset. doi: 10.15468/dmdck6 (accessed via GBIF.org 2023-01-24)

Stöver, B.C., Müller, K.F. (2010). TreeGraph 2: Combining and visualizing evidence from different phylogenetic analyses. BMC Bioinform. 2010, 11, 7. Retrieved from URL https://bmcbioinformatics.biomedcentral.com/track/pdf/10.1186/1471-2105-11-7.pdf

Stoyanov, S., Vladimirov, V. & Bancheva, S. (2021). *Sisyrinchium rosulatum* (Iridaceae), a new alien species to the Bulgarian flora. Abstracts Book (page 28), 1st International Conference on Botany and Mycology, Sofia, Bulgaria, 25–26 October 2021. Retrieved from URL https://icbotmyco.com/wp-content/

Stoyanov S, Vladimirov V, Bancheva S, Gogushev G (2023). *Sisyrinchium rosulatum* (Iridaceae), a new alien species to the Bulgarian flora. *BioInvasions Records*, 12(2), 493–500, doi 10.3391/bir.2023.12.2.13

Tacuatiá, L.O., Kaltchuk–Santos, E., Souza–Chies, T.T., Eggers. L., Forni–Martins, E.R., Pustahija, F., Robin, O. & Siljak–Yakovlev, S. (2017). Physical mapping of 35S rRNA genes and genome size variation in polyploid series of *Sisyrinchium micranthum* and *S. rosulatum* (Iridaceae: Iridoideae). *Plant Biosystems – An International Journal Dealing with all Aspects of Plant Biology*, 151(3), 403–413. doi 10.1080/11263504.2016.1179691

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673–4680. doi: 10.1093/nar/22.22.4673

Tirel, C., Jérémie J. & Lobreau–Callen, D. (1996). *Corchorus neocaledonicus* (Tiliaceae), véritable identité de l'énigmatique Oceanopapaver. *Bulletin du Muséum National d'Histoire Naturelle*, 18, 35–43. Retrieved from URL https://www.biodiversitylibrary.org/page/13740203#page/321/mode/1up

University of North Carolina at Chapel Hill Herbarium (NCU). University of North Carolina at Chapel Hill Herbarium: Vascular Plants. Occurrence dataset. doi: 10.15468/63vxjd (accessed via GBIF.org 31.01.2023)

Van Westen, M. Micam 2.4. Retrieced from URL http://science4all.nl

WFO: *Sisyrinchium micranthum* Cav. Retrieved from URL http://www.worldfloraonline.org/taxon/wfo-0000786480

Received: 21.03.2023
Accepted: 22.05.2023