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Solanum kieseritzkii and its comparison with *S. dulcamara* in Hyrcanian forests (North of Iran) based on morphological and molecular data

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Abstract

Solanum kieseritzkii, an endemic species of Iran and the Caucasus, has been recently introduced in botanical sources as unresolved species and sometimes has been regarded a synonymous of *S. dulcamara*. In this research, along with field and herbarium studies, a morphological and molecular comparison is made between these two species; each of them is designated as a distinct species. *Solanum kieseritzkii* is a creeping rhizomatous plant 10–30 cm high, has only 1–3 flowers per inflorescence, and is found only in the dark forests of Hyrcanian province, while *S. dulcamara* is a plant of open habitats including residential areas, climbing the tress up to 300 cm high, and has inflorescences with 6–40 flowers. The results of the present study were highly supported by comparison of internal transcribed spacer (ITS) sequences of different samples of both *S. kieseritzkii* and *S. dulcamara* and also comparing with other accessions from Genbank. Separation of two species is also confirmed by presented phylogenic tree.

Keywords: Distribution, endemic, habitat, phylogeny, *Solanaceae*, systematic

مطالعه *Solanum kieseritzkii* و مقایسه آن با *S. dulcamara* در جنگل‌های هیرکانی (شمال ایران) براساس داده‌های مورفولوژیکی و مولکولی*

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خلاصه

Solanum kieseritzkii C.A.Mey. یک گونه انحصاری ایران و قفقاز است که در منابع اخیر گیاه‌شناسی به عنوان گونه نامعین (unresolved) معرفی شده و اغلب با گونه *S. dulcamara* L. مترادف شده است. در این تحقیق، ضمن بررسی‌های میدانی و هرباریومی، مقایسه ریخت‌شناسی و مولکولی بین این دو گونه انجام شده و هر کدام به عنوان یک گونه مجزا اعلام می‌شوند. *S. kieseritzkii* تنها در کف جنگل‌های تاریک هیرکانی و به صورت خزنده و ریزوم‌دار مشاهده می‌شود که ارتفاع گیاه اصلی بین ۳۰-۱۰ سانتی‌متر و دارای ۳-۱ گل و میوه می‌باشد، در حالی که گونه *S. dulcamara* با تفاوتی قابل ملاحظه در مناطق باز و آفتابی و حتی در مناطق مسکونی، به ارتفاع تا ۳۰۰ سانتی‌متر روییده و از گیاهان و درختان دیگر به عنوان تکیه‌گاه استفاده می‌کند. همچنین، تعداد گل و میوه این گیاه بین ۴۰-۶ عدد می‌باشد. در پژوهش حاضر، علاوه بر بررسی مورفولوژیکی، توالی ژنتیکی دو نمونه از گیاه *S. kieseritzkii* و دو نمونه از گیاه *S. dulcamara* با استفاده از ناحیه فاصله‌انداز رونویسی شونده درونی (ITS)، تهیه، ثبت و با توالی‌های مشابه در بانک ژن NCBI مقایسه و بررسی شدند. نتایج حاصل از داده‌های مولکولی نیز حاکی از تمایز فیلوژنتیکی دو گونه مورد نظر است.

واژه‌های کلیدی: انحصاری، پراکنش، تیره سیب زمینی، زیستگاه، سیستماتیک، فیلوژنی

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Introduction

Solanum L. is the largest and most economically important genus of the family *Solanaceae*. It comprises about 1500 species worldwide (Weese & Bohs 2007) which many of them have been reported for medicinal properties and ethnobotanical uses (Eskandari *et al.* 2019). *Solanum kieseritzkii* C.A.Mey. is one of the rare species among the *Solanum* species which has been only found in the Hyrcanian forests in Iran and Azerbaijan (Fig. 1). Based on a study of diversity and distribution patterns, *S. kieseritzkii* with 0.953 SDI (Species Distribution Index), 0.333 RI (Rarity Index) and 1.287 CV (Conservation Value), has been identified as a rare species of *Solanaceae* in Iran (Sayadi & Mehrabian 2016). This species is a perennial plant usually growing in mountain forests. In Iran, we have observed it in Hyrcanian forest in 300–2300 m above sea level, where it grows on a wide range of geographical, climatic and soil conditions and thrive well even in infertile soils. Environmental factors including slope, orientation, silt percent, pH, organic matter and soluble phosphorous were among the most effective factors in establishment of this species (Mataji 2010).

Solanum kieseritzkii often were seen with *Ruscus hyrcanus* Woronow occurs at low- and mid-altitudes on northern slopes over calcic (somtimes non-calcic) parent rocks with deep, heavy-textured with drained soils. The soil is less acidic (pH=6–6.5) and rich in nitrogen, phosphorous, calcium and magnesium but moderate in potassium. The humus is less acidic and is often eutrophic and mesotrophic mull (Sagheb Talebi 2014). This plant often prefers shady, relatively light and wet areas and moves crawling on wetlands, and is one of the Solasodine-bearing species (Herbert 1973) as well. So far, it can be said that, no reliable references have yet been made on the biosystematics of this species. In contrast, *S. dulcamara* L. is widely distributed across Eurasia and northern North America (Fig. 2) where it is also common from sea level to ca. 2000 m. This weedy species grows in a wide variety of temperate habitats, often associated with water and open places with abundant light (Knapp 2013).

Solanum kieseritzkii was first described in 1831 by Meyer from shady damp woods of the lower mountain zone of the Lenkoran-Astara region in the Republic of Azerbaijan followed by its further reports in Flora Orientalis (Boissier 1875), Flora of the USSR (Pojarkova 1955), Flora of the Azerbaijan (Agajanov 1957), Flora Kavkaza (Grossheim 1967), Flora Iranica (Schonbeck-Temesy 1972), and *Solanaceae* in Flora of Iran (Khatamsaz 1998).

This species has been described in detail in the Flora of Iran (in Persian) along with a picture and distribution map (Khatamsaz 1998). In recent years, this species has been reported from several areas of Iran (Hyrcanian forests) in floristic and Phytosociological series of studies (Akhani 1998, Razavi & Esmailzadeh 2004, Mataji *et al*. 2007, Razavi 2008, Mataji *et al*. 2010, Assadi *et al*. 2011, Naqinezhad *et al*. 2012, Naqinezhad & Zarezadeh 2012, Adel *et al.* 2014, Bazdid Vahdati *et al*. 2014, Mataji *et al*. 2014, Akhondnejad *et al*. 2016, Mirzaei *et al*. 2016, Moradi *et al*. 2016, Deljouei *et al*. 2017).

In some references, *S. kieseritzkii* has been introduced as synonymy of *S. dulcamara* (http://solanaceaesource.org). According to Knapp (2013), *S. kieseritzkii* is given to a variety of different samples of *S. dulcamara* with small erect shoots connected with creeping stems with only a few flowers on each having very small inflorescences.

In the present study, we aimed to illuminate the taxonomic status of *S. kieseritzkii* using critically morphological examination of extensive collections from North of Iran and Azerbaijan by phylogenetic analyses of the DNA sequence data of ITS region of the nuclear ribosomal DNA.

Materials and Methods

- Field survey and Herbarium studies

In order to assess the morphological traits of *S. kieseritzkii*, over 35 localities of *S. kieseritzkii* were

visited in the Hyrcanian forests and/or reviewed in the herbarium specimens. Our circumscription of this species is based on herbarium specimens study at GUM (University of Guilan, Rasht, Iran), IRAN (Iranian Research Institute of Plant Protection, Tehran, Iran), TARI (Research Institute of Forests and Rangelands, Tehran, Iran), TMRC (Shahid Beheshti University of Medical Sciences, Tehran, Iran), TUH (Tehran University, Tehran, Iran), and W (Natural History Museum, Vienna, Austria) herbaria (Table 1). All of these herbaria have been introduced in Index Herbarium (Thiers 2016). The distribution map was prepared using specimens with verified identity and also with geographical coordinate data on the labels (Figs 1 & 2). It should be noted that, unlike *S. kieseritzkii*; *S. dulcamara* is a cosmopolitan species and is found in most of central and northern parts of Iran.

Table 1. List of *Solanum kieseritzkii* visited in Hyrcanian forests and reviewed in herbaria specimens

Locality	Country	Latitude	Longitude	Date	Altitude (m)	Collector	Herbarium No.
Gilan prov.: 10 km SE Lahijan, Ata-Kuh forest	Iran	37.144	50.081	-	300	Bazdid Vahdati	GUM 4172
Gilan prov.: Asalem to Khalkhal forest	Iran	37.683	48.838	15.07.1975	1000	Wendelbo/Assadi	TARI 18380
Gilan prov.: Asalem to Khalkhal forest	Iran	37.65	48.817	29.05.1978	800	Wendelbo/Assadi	TARI 27726
Gilan prov.: Asalem to Khalkhal forest	Iran	37.672	48.812	09.11.1994	1500	Khatamsaz/Farzaneh	TARI 73151
Gilan prov.: Asalem to Khalkhal road to Piceson Nursery	Iran	37.676	48.773	06.07.2013	1250	Mozafarian	TARI 1022414
Gilan prov.: Asalem to Khalkhal, Almas, Shahgerdekuh, Kale Kale forest	Iran	37.675	48.733	06.08.2013	1434	Mozafarian	TARI 102556
Gilan prov.: Deylaman to Siahkal	Iran	36.931	49.903	07.06.2011	1300	Noroozi, J.	W 2011-0012285
Gilan prov.: Talesh, Asalem	Iran	37.705	48.889	-	-		W 1967-18775
Gilan prov.: Talesh, Asalem	Iran	37.705	48.889	01.04.1966	-	Tregubov	TUH 190794
Golestan prov.: Bandar Gaz	Iran	36.736	54.017	28.051948	-	Sharif	IRAN 40470
Golestan prov.: Closed montane forest on steep, northern slopes of Alu-Baq (South of Tangegol)	Iran	37.367	55.933	1998	1450	Akhani	W 1999-07548
Golestan prov.: Gorgan forest	Iran	36.79	54.464	06.06.1956	300	Schmidt	W 1959-0024347
Golestan prov.: Gorgan, Shamushak forest	Iran	36.728	54.235	16.07.2017	700	Eskandari/Bahramishad	IRAN 74537
Golestan prov.: Gorgan, Shamushak forest	Iran	36.728	54.235	06.05.2018	1026	Bakhshi/Bahramishad	IRAN 75644
Golestan prov.: Loveh to Gonbad-e Kavous	Iran	37.360377	55.658044	18.05.1968	-	Tregubov	TUH 190795
Mazandaran prov.: Galugah, Niala	Iran	36.688	53.774	30.04.2016	900	Eskandari/Bahramishad	IRAN 74453

Table 1 (contd)

Mazandaran prov.: Amol, Chamestan, Lavij to Mirkhamand (forest)	Iran	36.327	52.009	12.10.2017	2000	Eskandari/Bakhshi/ Ghamghami	IRAN 74792
Mazandaran prov.: Chalus, Veysar	Iran	36.463	51.542	09.10.1977	500	Ronemak/Mozafarian	TARI 25904
Mazandaran prov.: Galanderoud	Iran	36.446	51.907	15.04.1959	-	Sabeti	IRAN 40473
Mazandaran prov.: Kelardasht	Iran	36.494	51.126	08.06.1991	-	Izadpanah	TARI 59817
Mazandaran prov.: Kheyrrud-Kenar forest	Iran	36.597	51.576	22.06.1980	500	Assadi	TARI 33461
Mazandaran prov.: Noshahr, 5 km after Nowshahr to Nur, Kheyrud-Kenar forest	Iran	36.605	51.571	07.05.2008	-	Moazzeni	TMRC 0001518
Mazandaran prov.: Noshahr, Kheyroud-Kenar forest	Iran	36.615	51.536	02.08.2008	1200	Moradi/Siadati	TUH 40032
Mazandaran prov.: Nowshahr	Iran	36.617	51.477	12.09.1956	-	Esfandiari	IRAN 40471
Mazandaran prov.: Nowshahr, SE Bandpey	Iran	36.604	51.582	30.08.1974	800	Wendelbo/Assadi	TARI 14579
Mazandaran prov.: Nowshahr, Kheyroud-Kenar forest	Iran	36.614	51.537	-	-		W 1960 10976
Mazandaran prov.: Nowshahr, Kordi Chal	Iran	36.537	51.222	22.09.1956	-	Sabeti	IRAN 40472
Mazandaran prov.: Ramsar, Mazibon and Sibon protected forest	Iran	36.893	50.616	2010	1000	Naqinezhad	GUM 14269
Mazandaran prov.: SE Bandepey, above microwave station	Iran	36.601	51.586	30.08.1974	800	Wendelbo/Assadi	W 1976-0003420
Mazandaran prov.: SW Tonekabon, Liresar, Lesakooti forest	Iran	36.78	50.722	14.07.1990	1300	Hamzeh'ee	TARI 71017
Mazandaran prov.: Sangdeh, above Talar-e Sarband	Iran	36.023	53.223	18.06.1995	2300	Assadi	TARI 73396
Mazandaran prov.: Veissar	Iran	36.463986	51.542517	17.05.1965	-	Tregubov	TUH 190793
Mazandaran prov.: Amol, Chamestan to Vaz, Jurband to Vaz Tangeh	Iran	36.390833	52.106944	28.05.2019	-	Pahlevani/Torabi	IRAN 76558
Mazandaran prov.: Nowshahr, Salahedinkola, Mollakola village	Iran	36.5225	51.779722	29.05.2019	-	Pahlevani/Torabi	IRAN 76557
In montibus sylvaticis [circa aquas calidus] prope Lenkoran, [locis umbrosis subhumidis]	Azerb-aijan	38.754132	48.753747	23.05.1830	-	C.A.Mey.	LE 00016961

Fig. 1. Distribution of *Solanum kieseritzkii* in the world and Iran.

Fig. 2. Distribution of *Solanum dulcamara* in the world and Iran.

- Morphological studies

For the comparison of *Solanum kieseritzkii* with the closely related species *S. dulcamara*, we used 20 morphological traits including six qualitative and 14 quantitative characters (Table 2).

- Molecular studies

For molecular analyses, we used the Internal Transcribed Spacer (ITS) DNA sequence data. For this purpose, four specimens including two samples of *S. kieseritzkii* (IRAN 74537 and IRAN 40473) and two samples of *S. dulcamara* (IRAN 74599 and IRAN 74782) were subjected to molecular studies in order to compare with other *Solanum* species. Silica- gel dried leaves of the plants collected from the field were used for source of DNA extraction using CTAB modified protocol (Porebski *et al.* 1997). Molecular method and protocols is treated after Younesi *et al*. (2016).

The ITS region was amplified by using ITS1 (5′ - TCCGTAGGTGAACCTGCGG - 3′) and ITS4 (5′ - TCCTCCGCTTATTGATATGC - 3′) primers (Douzery *et al.* 1999). PCR reaction in total volume of 30 µl containing 100 ng of gDNA, 0.8 µm each primers, 1.5 mM MgCl2, 15 µl of 2x *Taq* DNA polymerase mix (Ampliqon, Denmark) carried out following thermo-cyling program: initial denaturation at 95 °C for 8 min, 35 cycles of 94 °C for 30 s, 56 °C for 50 s, 72 °C for 60 s, and final extension of 72 °C for 15 min. Quality control of PCR reactions checked on 1% agarose gel electrophoresis and purified in Expin Combo kit (GeneAll, Korea). PCR products were sequenced on ABI 3730xl in two directions (Macrogen Co, Korea).

- Phylogenetic analysis

DNA sequence data were visually checked and edited in Sequencher Ver. 4 (Gene Codes Corporation,

Ann Arbor, Michigan, USA) software. The newly generated sequences during this study were compared with sequences available in National Center for Biotechnology Information (NCBI) GenBank nucleotide database using a megaBLAST search. The obtained sequences from GenBank together with our sequences were aligned with MAFFT Ver. 7 online interface using default settings (http://mafft.cbrc.jp/alignment/server) (Katoh & Standley 2013). The best- fitting model of DNA nucleotide substitution was discovered using MrModeltest Ver. 2.3 (Nylander 2004). A Bayesian phylogenetic reconstruction was performed with MrBayes Ver. 3.2.6 (Ronquist *et al.* 2012) based on the results of MrModeltest as explained by Bakhshi (2018). The heating parameter was set at 0.15 and burn-in was set to 25% and trees were saved each 1000 generations. The Markov Chain Monte Carlo (MCMC) analysis of four chains was started in parallel from a random tree topology and lasted until the average standard deviation of split frequencies reached a value of 0.01 (stopval=0.01). The resulting phylogenetic tree was printed with Geneious Ver. 8.1.8 (Kearse *et al.* 2012). All the new sequences generated in this study, were deposited in NCBIs GenBank nucleotide database (www.ncbi.nlm.nih.gov).

Results and Discussion

- Morphology and field survey

Along herbarium studies, a morphological comparison is made between these two species, each of which is designated as a distinct species. The results of morphological studies are presented in table 2. During the comparison of the twenty different characters between these two species, only in one case (corolla color) the same property was observed, and in the remaining cases there were a differences. Based on field observations *S. kieseritzkii* is found and prefers shady, relatively light and wet areas in forests, creeping with rhizome, the height of the plant varies from 10–30 cm, that has only 1–3 flowers and fruits, while *S. dulcamara* has a height of 300 cm and climb on other trees as a support, it grows in open and sunny habitats and even in residential areas; the number of flowers and fruits varies from 6–25.

- Phylogenetic analysis

The final alignment of the ITS consisted of 388 characters (including the alignment gaps), representing 17 sequences of the genus *Solanum* (including 13 sequences from NCBI and four sequences from this study), and *Nierembergia linariifolia* R. Grah. (GenBank accession number AY560055) as an outgroup.

Based on the results of MrModel Test, the Bayesian analysis is performed with the SYM+G substitution model, with gamma rates and fixed frequencies. The alignment contained a total of 75 unique site patterns. The Bayesian analysis lasted 1290000 generations and saved a total of 2582 trees. After discarding the first 25% of sampled trees for burn-in, the consensus trees and posterior probabilities (PP) were calculated from the remaining 1938 trees and the final tree is depicted (Fig. 6).

Based on the phylogenetic analyses of the ITS locus, *Solanum kieseritzkii* and *S. dulcamara* were grouped in two different clades (Fig. 6). Finally, as a result drawn by the morphological and molecular data, we hereby treated *S. kieseritzkii* as a distinct species.

One of the systematic problems that have occurred in recent years with regard to *Solanaceae* family in Iran is that, many species reported in Flora Iranica from Iran, were synonyms with other species or introduced as unresolved species by some researchers. For instance, it seems that, one of the reasons for considering *S. kieseritzkii* and *S. dulcamara* as synonyms (Knapp 2003) is because it has been studied on herbarium-based specimens only. It is clear that, although both species are similar in their herbarium sheets, but they have very distinct in their natural habitat resulting in clear separation with different morphological features.

Separation of a taxon from two distinct taxa is a step towards realizing biodiversity in the form of

scientifically introduced or interpreted taxonomic-systematic review of a species causing managerial attention rather than focusing on one species on two distinct species. Therefore, this study is an important step towards more effective conservation of biodiversity and plant species diversity.

- Taxonomy

Solanum kieseritzkii C.A.Mey., Verz. Pfl. Cauc. 113. 1831 Ldb. Fl. Ross. IH, 188; Dun. in DC. Prodr. XIH, 1, 78; Boiss. Fl. or. IV, 285; Grossh. Fl. Kavk. HI, 355. Type: Azerbaijan. In montibus sylvaticis circa aquas calidus prope Lenkoran, locis umbrosis subhumidis, 23 May 1830, C.A.Mey. s.n. [type specimens: LE]; Herbarium Russian Academy of Sciences-V.L. Komarov, Botanical Institute (LE), LE00016959.

Since accession to type specimen was not available, therefore, two *S. kieseritzkii* herbarium samples that were collected from type locality and are authentically named are given in figure 7.

Table 2. Morphological differences of typical *Solanum kieseritzkii* and *S. dulcamara* based on field observations and herbarium specimens

No.	Character	*S. kieseritzkii*	*S. dulcamara*
1	Growth habit	Prostrate & climbing	Climbing
2	Stem	Glabrous	Hairy or subglabrous
3	Corolla color	Light purple	Purple
4	Berry color and shape	Dark red, globose	Shiny red, ovoid or ellipsoid
5	Seed shape	Flat, orbicular-reniform	Orbicular-reniform
6	Leaf shape	Elliptical or lanceolate	Ovate or lanceolate
7	Plant height (cm)	10–30	200–300
8	Number of flowers per inflorescence	1–3	6–40
9	Peduncle length (mm)	3–10	10–50
10	Pedicle length (mm)	7–17	6–15
11	Calyx length (mm)	1.5–2.5	2–2.5
12	Corolla length (mm)	8–10	7–15
13	Berry diameter (mm)	10–12	5–10
14	Number of berries per inflorescence	1–3	6–40
15	Seed length (mm)	3.5–4.5	2.5–3
16	Seed width (mm)	2.5–3.5	2.5–3
17	Lamina length (cm)	11–14	5–9
18	Lamina width (cm)	6.5–8	2.5–5
19	Petiole length (cm)	1/4–1/3	1/3–1/2
20	Anthers length (mm)	3.5–5	4.5–6

Fig. 3. *Solanum kieseritzkii* (Hand-drawing).

Fig. 4. *Solanum kieseritzkii* (above) and *S. dulcamara* (below) habit.

Fig. 5. Fruit and seed of *Solanum kieseritzkii* (left) and *S. dulcamara* (right).

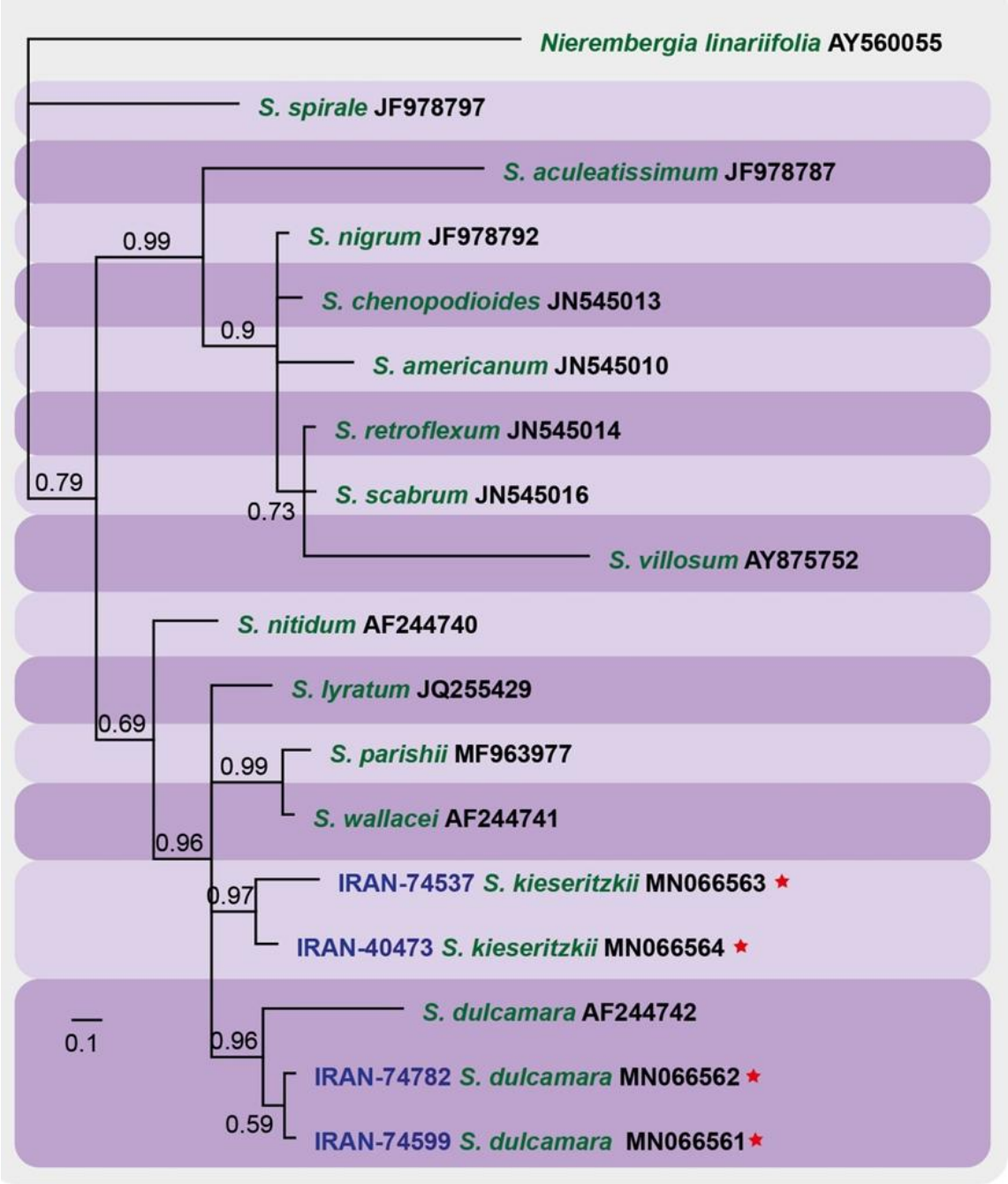

Fig. 6. Consensus phylogram of studied *Solanum* species (50% majority rule) of 1938 trees resulting from a Bayesian analysis of the ITS sequence alignment using MrBayes Ver. 3.2.6. The scale bar indicates 0.1 expected changes per site. The tree was rooted to *Nierembergia linariifolia* (AY560055). The sequences obtained in this study are shown by asterisk.

Fig. 7. *Solanum kieseritzkii* (authentically named material from type locality) in HBG (left) & W (right).

- Description

For description of the species, we used herbarium plants and other sources such as Flora Iranica (Schonbeck-Temesy 1972) and *Solanaceae* in Flora of Iran (Khatamsaz 1998).

Stems short, simple, with closely spaced branches, ascending to 10–30 cm. Rhizome woody, branched, brown, slender and long, up to 2 m long or even more. Leaf blades entire, lanceolate, few, thin (dry ones chartaceous), subglabrous, bright green above, pale beneath, up to 11 cm long and 6.5 cm broad, from elliptical-ovate, sharply tapering and mucronate at the apex to elliptical and narrowly elliptical-lanceolate, long accuminate, entire, with cuneate base, decurrent on slender petiole 1/4–1/3(1/2) as long as lamina. Flowers 1–2(3) in terminal bostryx with short peduncle (mostly shorter than pedicel). Peduncle 3–10 mm long. Pedicel recurved, slender, 7–17 mm long, thickened above. Calyx glabrous, broad, shallowly 5-lobed or dentate with broad triangular lobes or teeth, entire. Corolla light purple, 16–20 mm with 5 pairs of green spots, 5-lobed; lobes triangular-lanceolate and deflexed, with white cilia along the margin and on the outside at the tip. Anthers linear, 3.5–5 mm long, free or connate in middle, conically connivent or partly connate, dehiscing by two apical pores, later transforming into short slits. Style thin, longer than stamens. Berry about 1 cm in diameter, globose, dark red, blackish. Seeds flat, orbicular-reniform, about 3.5 mm long and wide.

To show more details, herbarium sample were illustrated in figure 3 and photographs of the habitat, flowers, fruits and seeds of both species were shown in figures 4 & 5.

Flowering season: June to July

Fruiting season: August to September

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