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Morphological and molecular determination of a new *Viola* species (Violaceae) from Turkey

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Abstract

Viola denizliensis is described as a new species from South-West Anatolia. The species grows on serpentinite stony slopes in Bozdağ Mountain in Denizli. It belongs to *Viola* sect. *Melanium* and is close to *V. dirimliensis* and *V. kitaibeliana*. *Viola denizliensis* can be readily distinguished from related species by morphological and molecular characters. Comments on descriptive and diagnostic characters, distribution and ecology, phenolgy and proposed conservation status for this new species are given in the present study. Morphological affinities and ISSR-based molecular relationships between *V. denizliensis* and related species are also discussed.

Keywords: Anatolia, endemics, ISSR, *Melanium*, systematics, taxonomy

Introduction

The Violaceae are a medium-sized cosmopolitan family containing 22 recognized genera and 1000−1100 species of herbs, shrubs, lianas and trees (Wahlert *et al.* 2014). While the Violaceae family was placed in the Violales in previous classification systems (cf. Cronquist 1981), more recent classifications place it in Malpighiales (cf. Angiosperm Phylogeny Group: APG 2016).

Viola Linnaeus (1753: 933), the type genus of Violaceae, includes about 600 mostly herbaceous species, occurring throughout most temperate regions of the World (Mehrvarz *et al.* 2013, Zhou *et al.* 2008). Some *Viola* species are perennial, some of them are annual, and a few are small shrubs. Many species, varieties and cultivars of *Viola* are grown as ornamental flowers in gardens. *Viola* is represented by 35 taxa (33 species plus 2 subspecies) in Turkey so far (Blaxland 2004, Coode & Cullen 1965, Davis *et al.* 1988, Dinç 2012, Dinç *et al.* 2000, 2001a, 2001b, 2003, Dinç & Yıldırımlı 2002, Knoche & Marcussen 2016, Marcussen &Borgen 2011, Yıldırımlı 1994, 2000, Yıldırımlı & Dinç 2002).

During field work of the project "Biodiversity and Monitoring studies of Terrestrial and Inland Water Ecosystems in Denizli Province" in a expedition in April 2017, some interesting specimens of an unknown member of *Viola* were found in Bozdağ Mountain, Denizli province (southwestern Anatolia, Turkey). On a further visit to the same locality in May 2017, additional material was gathered providing a range of specimens bearing good flowers and fully mature fruits. Detailed morphological and molecular studies based on Inter Simple Sequence Repeat (ISSR) genetic relationships revealed that those specimens belong to a new species which is described here as *V. denizliensis*.

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Material and Methods

Morphological study:—A total of 20 herbarium specimens of the new species were collected from the type locality. Specimens were prepared for morphological and molecular phylogenetic studies according to standard herbarium techniques and preserved in the Pamukkale University Herbarium (PAMUH). After drying process, these specimens were checked using the basic floras, Flora of Turkey (Coode & Cullen 1965, Davis *et al.* 1988, Yıldırımlı 2000), Flora europaea (Valentine *et al.* 1968) and related papers (Blaxland 2004, Dinç 2012, Dinç *et al.* 2000, 2001a, 2001b, 2003, Dinç & Yıldırımlı 2002, Knoche & Marcussen 2016, Marcussen & Borgen 2011, Yıldırımlı 1994, Yıldırımlı & Dinç 2002), and also confirmed by comparison with digital images of herbarium samples in the herbaria E, K, W, JE and P.

Molecular study:—DNA was extracted using a modified method based quick DNA extraction protocol without liquid nitrogen (Ferdous *et al.* 2012) from dried *Viola* herbarium samples. This modification includes only an extra step of chloroform:isoamyl alcohol:phenol (24:1:5%) treatment. PCR reactions were performed in a 25 µl reaction mix (1xPCR Buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTP and 1Unit Taq DNA polymerase) using 40 ng/µl DNA template and ten ISSR primers [ISSR1 $(AG)_8T$ (GenBank accession number, UBC807); ISSR2 $(AG)_8G$ (UBC809); ISSR3 $(GA)_8T$ (UBC810); ISSR4 $(GA)_8C$ (UBC811); ISSR5 $(CA)_8A$ (UBC817); ISSR6 $(TC)_8C$ (UBC823); ISSR7 $(AC)_8C$ (UBC826); ISSR8 $(AG)_8CTT$ (UBC846); ISSR9 $(CA)_8AGT$ (UBC855); ISSR10 $(GT)_8CTA$ (UBC856), Martins-Lopes *et al*. 2009, Smykal *et al.* 2011)]. Thermocycler conditions, for 3 min at 95 ºC for initial denaturation was followed by 35 cycles for 15 sec at 95 °C, 30 sec at 55 °C, 3 min at 72 °C and final extension completed with 10 min at 72 ºC. Gel electrophoresis was used for separation of PCR products in 1.5% agarose gel and band profiles dyed with ethidium bromide, visualized under UV light, and monitored with image analysis system. DNA band profiles of ISSR PCRs were scored by their absence (0) or presence (1), and the ones at low density were scored only if they were reproducible in both the PCR runs. Cluster analysis was performed to construct dendrograms of the distance trees, with the unweighted pair-group method by arithmetic averages (UPGMA) from the similarity data matrices using Jaccard's coefficient (D-UPGMA, 2002).

Results

Viola denizliensis O.D.Düşen, Göktürk, U.Sarpkaya & B.Gürcan, *sp. nov.* (Fig. 1–2)

Type:—TURKEY. C2 Denizli: Nikfer, Bozdağ Mountain, serpentinite stony slopes, 1670 m, 29 April 2017, *O.D.Düşen 1512* & *R.S.Göktürk* (holotype: PAMUH!; isotypes: Akdeniz University Herbarium!).

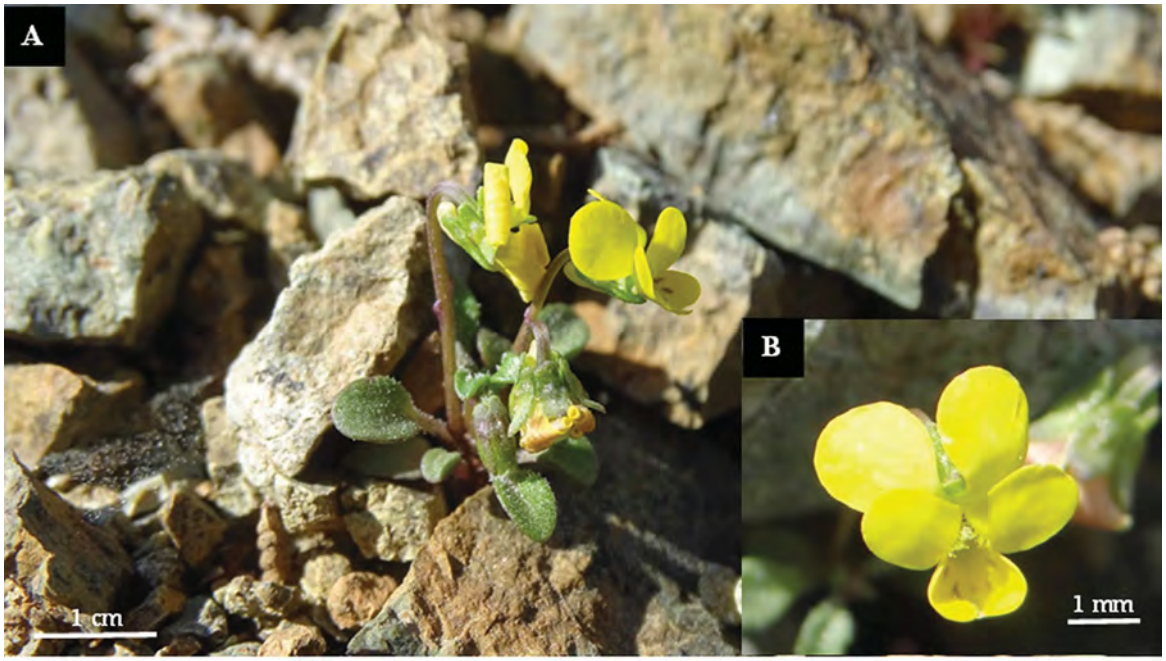

FIGURE 1. *Viola denizliensis* O.D.Düşen, Göktürk, U.Sarpkaya & B.Gürcan, *sp. nov.* **A)** General view in nature; **B)** Close-up wiew of flower.

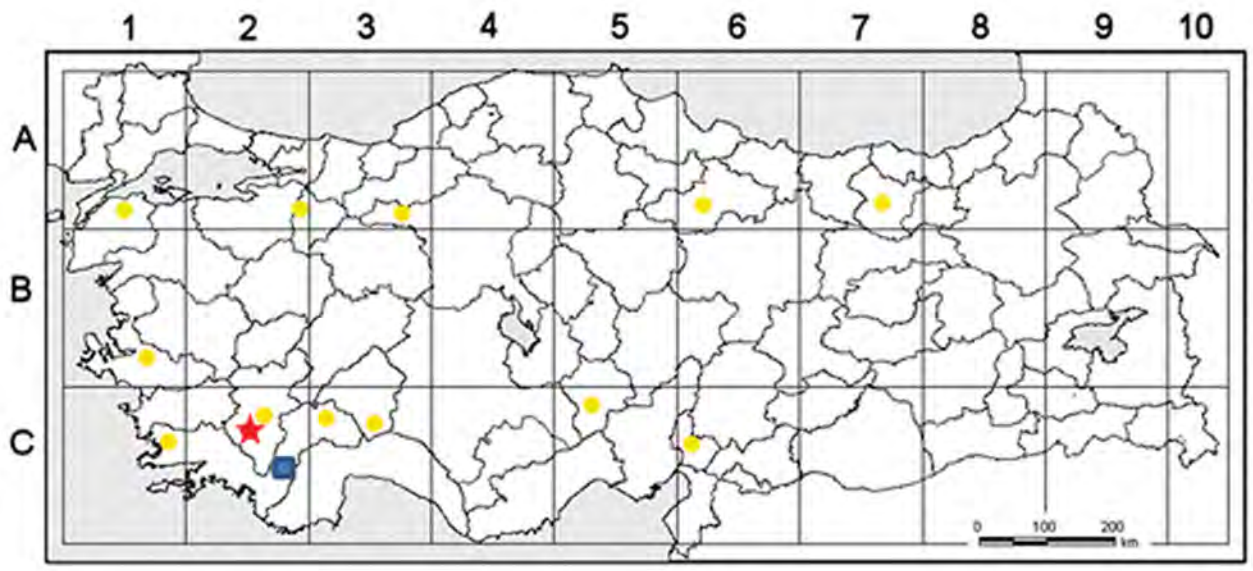

FIGURE 2. Known distribution of *Viola denizliensis* (red star), *V. dirimliensis* (blue square) and *V. kitaibeliana* (yellow dots) in Turkey.\

Description:—Very tiny annual plant, 0.5–2.7 (–3.0) cm high at flowering. Taproot usually upper part dark purple, lower part colourless and not branching. Stem very short, greenish to greenish-purple, simple or branching, erect or ascending, glabrous. Leaves usually mostly at base, sometimes scattered on stem, elliptic to spathulate, entire, tip obtuse, base attenuate, long-decurrent on the petiole, with elongate, retrorse hairs on margin. Lamina upper surface hirsute, lower surface glabrous, purplish. Stipules entire, undivided, linear, much smaller and shorter than the leaf, hirsute. Peduncles greenish to greenish-purple, glabrous, up to 1.5 cm long, longer than leaves. Bracteoles distinct, on the upper third of the peduncle, greenish to greenish-purple, translucent, glabrous, adpressed to the peduncle, 1.0 mm long Flowers not fragrant, very small, up to 4.0 mm long including spur. Sepals glabrous, with a prominent mid-vein, parallel sides and acute tip, 2.0 mm long; appendices green, glabrous, obtuse, undivided, extrorse, almost equal in length, 0.5–0.8 mm long. Petals unequal in size, 2.0–4.0 × 1.5–3.5 (–3.8) mm, golden-yellow; upper 2 petals largest, obovate; lateral 2 petals ovate, pointing upwards, with yellow hairs on the adaxial surface; the lowest petal small, concave, emarginated at apex, with a few and very short blackish-purple lines; spur yellow, small, flattened, glabrous, the apex rounded and turned upwards, longer than sepal appendices. Capsule globose, up to 2.0 mm in diameter, glabrous. Seeds 1.2 × 0.6 mm long, elliptical, brown, with a small colourless elaiosome.

Distribution and ecology:—*Viola denizliensis* is endemic to South-West Anatolia, Turkey (Fig. 2). It grows in serpentinite stony slopes at an elevation of 1670 m (Fig. 3). It is associated with other endemics such as *Verbascum trapifolium* (Stapf 1885: 88) Huber-Morath (1973: 16) var. *flabellifolium* (Huber-Morath 1973: 12) Karavelioğulları & Aytaç (2008: 17), *Eryngium thorifolium* Boissier (1844a: 122), *Centaurea drabifolia* Smith (1813: 202) subsp. *austro-occidentalis* Wagenitz (1963: 162), *Astragalus pelliger* Fenzl (1842: 5) and non-endemic plants such as *Pinus nigra* J.F.Arnold (1785: 8) subsp. *pallasiana* (Lambert 1828: 2) Holmboe (1914: 29) var. *pallasiana*, *Fritillaria pinardii* Boissier (1846: 106), *Tulipa armena* Boissier (1859: 99) var. *armena* and *Salvia frigida* Boissier (1844b 10).

Phenology:—Chasmogamous flowering from April to May, followed by cleistogamous flowering throughout the rest of the growing season. Fruiting from May to June.

Proposed conservation status:—*Viola denizliensis* is known only from one locality with small populations in Bozdağ Mountain. It is suggested that this new species should be placed under the IUCN threat category "Critically Endangered (CR)" (IUCN 2012), because the estimated area of occupancy is less than 10 km^2 (criterion B2) and it is known only from one locality (criterion B2a). The population size of the new species is estimated to be less than 50 mature individuals (criterion C2a-i). The population size of the new species could be reduced in the near future based on road construction between Nikfer to Bozdağ Mountain.

Etymology:—The specific epithet is derived from type locality.

Additional specimens examined of ***V. denizliensis*** (paratype):—TURKEY. C2 Denizli: Nikfer, Bozdağ Mountain, serpentinite stony slopes, 1670 m, 20 May 2017, *O.D.Düşen 1802* & *R.S.Göktürk* (PAMUH, Akdeniz Univ. Herb.).

FIGURE 3. Habitat of *Viola denizliensis*. **A)** Population on serpentinite stony slopes; **B)** Very tiny samples (marked with arrows) among serpentinite stones.

Other specimens examined:—***Viola dirimliensis*:**—TURKEY. C2 Burdur: Dirmil pass, serpentinite rocks, 1500-1600 m, 03 May 2011, *C.Aykurt (3061) & I.G.Deniz* (Akdeniz Univ. Herb.). ***Viola kitaibeliana*:**—TURKEY. C2 Denizli: Honaz Mountain National Park, *Pinus brutia* var. *brutia* clearings, 1215 m, 30 April 2017, *O.D.Düşen (1557)* & *R.S.Göktürk* (PAMUH). C2 Denizli: Denizli to Serinhisar, Kazıkbeli, stony places, 1181 m, 30 April 2017, *O.D.Düşen (1613)* & *R.S.Göktürk* (PAMUH).

Molecular phylogenetic relationships:—*Viola denizliensis* was compared with *V. dirimliensis* and *V. kitaibeliana* using cluster analysis of molecular data from ten ISSR primers. The total of 98 reproducible bands, ranging from 560 to 2300 bp, were obtained from ISSR1 (23), ISSR4 (28), ISSR5(25), and ISSR6 (22) and rates of polimorphism between *V. denizliensis* and *V. dirimliensis*, between *V. denizliensis* and *V. kitaibeliana*, between *V. dirimliensis* and *V. kitaibeliana* were 76.1% , 85.7% , and 74.6 %, respectively (Fig. 4, 5).

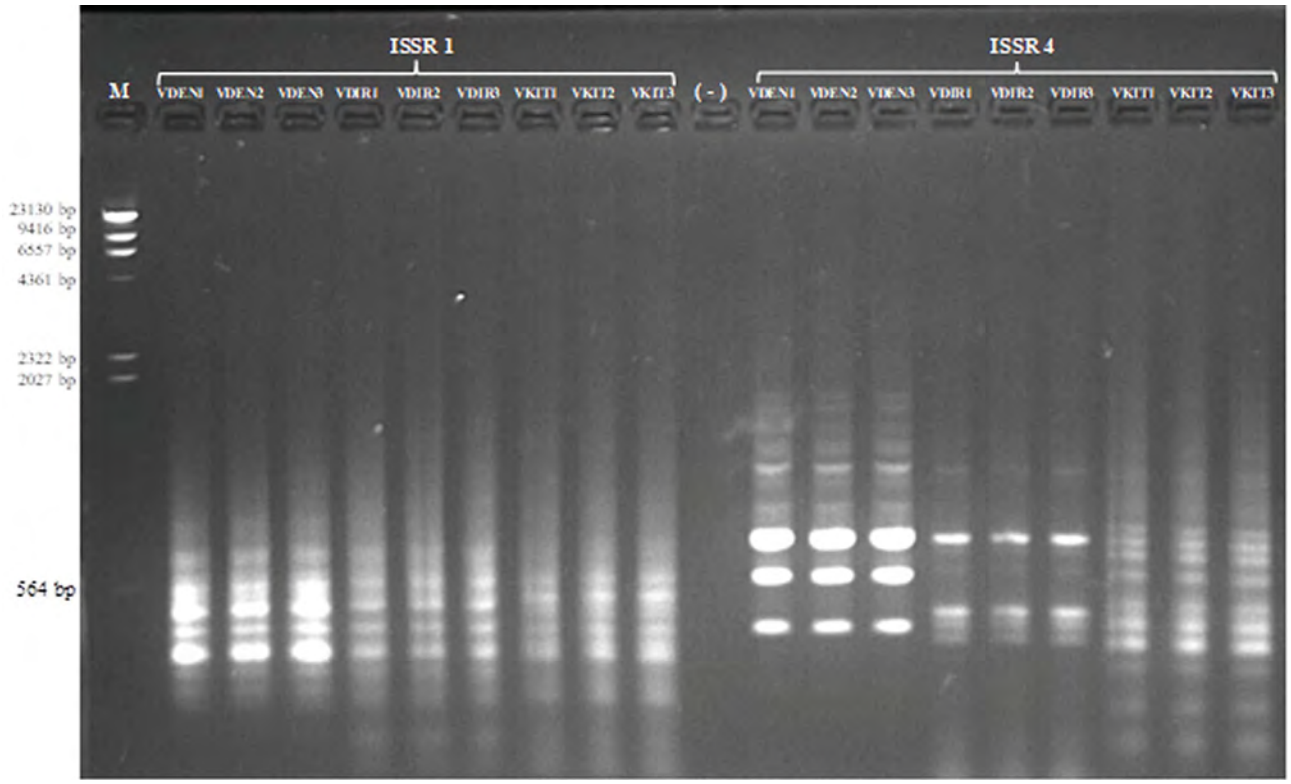

FIGURE 4. ISSR1 and ISSR4 PCR amplification products visualized with dying with ethidium bromide in 1.5% agarose gel for three different *Viola* taxa (VDEN1-3, *V. denizliensis*; VDIR1-3, *V. dirimliensis*; VKIT1-3, *V. kitaibeliana*; M, Lambda DNA/Hind III marker; (-) negative control PCR reaction was performed without using DNA template).

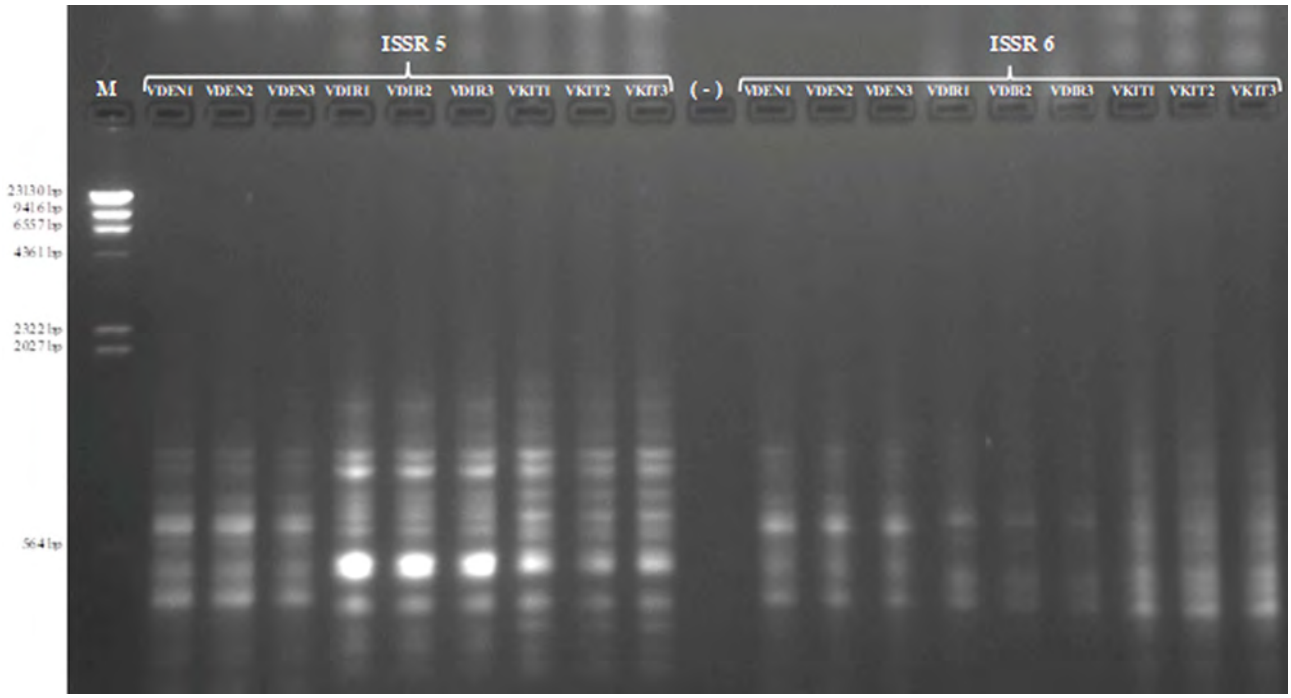

FIGURE 5. ISSR5 and ISSR6 PCR amplification products visualized with dying with ethidium bromide in 1.5% agarose gel for three different *Viola* taxa (VDEN1-3, *V. denizliensis*; VDIR1-3, *V. dirimliensis*; VKIT1-3, *V. kitaibeliana*; M, Lambda DNA/Hind III marker; (-) negative control PCR reaction was performed without using DNA template).

In order to calculate genetic distances of three different *Viola* taxa, the similarity matrix was generated by the Jaccard's coefficient method (Table 1). This matrix showed similarity rate ranging up to 0.143 among the same taxa samples, on the other hand similarity rate ranging from 0.579 to 0.692 among different *Viola* taxa. The phylogenetic tree of three diferent *Viola* taxa belonging to nine samples was constructed using UPGMA dendogram analysis program (Fig. 6).

The dendogram indicated that three *Viola* taxa divided into two main clusters with similarity range of 0.692. *Viola denizliensis* and *V. dirimliensis* were in the same main cluster but they further grouped two into different subclusters with similarity range from 0.579 to 0.597. On the other hand, *V. kitaibeliana* was divided into different main cluster and very far from the other two taxa with similarity range up to 0.692 (Fig. 6). This results were congruent with the morphological analysis.

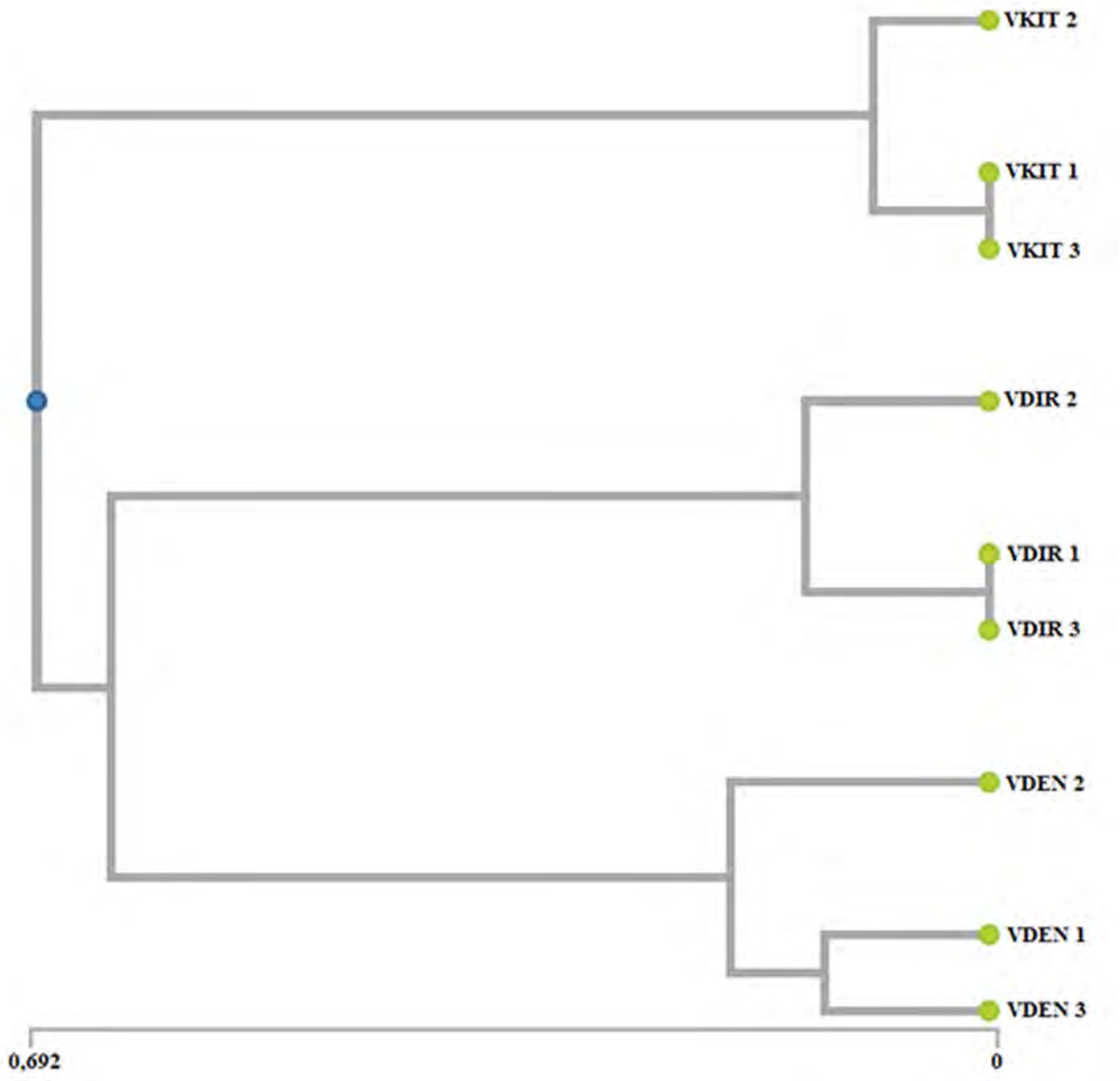

FIGURE 6. Dendogram of the nine samples belonging to three studied taxa of *Viola* using UPGMA cluster analysis based on genetic similarities of DNA fingerprinting patterns from the four ISSR primers (VDEN1-3, *V. denizliensis*; VDIR1-3, *V. dirimliensis*; VKIT1-3, *V. kitaibeliana*). The scale at the base indicates genetic distances.

TABLE 1. Similarity matrix generated by Jaccard's coefficient method (VDEN1-3, *V. denizliensis*; VDIR1-3, *V. dirimliensis*; VKIT1-3, *V. kitaibeliana*).

	VDEN1	VDEN2	VDEN3	VDIR1	VDIR2	VDIR3	VKIT1	VKIT2	VKIT3
VDEN1	0	0.143	0.117	0.579	0.585	0.579	0.692	0.687	0.692
VDEN2		0	0.143	0.585	0.591	0.585	0.687	0.682	0.687
VDEN3			0	0.591	0.597	0.591	0.682	0.677	0.682
VDIR1				0	0.083	0.000	0.591	0.597	0.591
VDIR2					0	0.083	0.597	0.603	0.597
VDIR3						0	0.591	0.597	0.591
VKIT1							0	0.083	0.000
VKIT2								0	0.083
VKIT3									0

Discussion

Although many literature (Blaxland 2004, Coode & Cullen 1965, Davis *et al.* 1988, Dinç, 2012, Dinç *et al.* 2000, 2001a, 2001b, 2003, Dinç & Yıldırımlı 2002, Knoche & Marcussen 2016, Marcussen & Borgen 2011, Yıldırımlı 1994, 2000, Yıldırımlı & Dinç 2002, Valentine *et al.* 1968) and herbarium samples were checked, samples collected in Bozdağ Mountain were consistently different from any other annual known taxa in the genus.

Viola denizliensis belongs to *Viola* sect. *Melanium* and is morphologically close to *V. dirimliensis* and *V. kitaibeliana*. The new species can be readily distinguished by morphological and molecular characters from those species (Fig. 6, Table 2). Furthermore, *V. dirimliensis* is endemic to Dirimli pass between Muğla and Burdur city border and grows serpentinite rocks. *Viola kitaibeliana* is largely distributed in Turkey and grows several habitats such as stony slopes, screes, macchie and banks.

TABLE 2. Morphological comparison of *Viola denizliensis* with *V. dirimliensis* and *V. kitaibeliana.* Data on both latter taxa are taken from literature (Blaxland 2004, Coode & Cullen 1965).

Characters	***Viola denizliensis***	***Viola dirimliensis***	***Viola kitaibeliana***
Lower Part of Taproot	unbranching	branching	branching
Stem	0.5–2.7 (-3.0) cm; simple or branching; glabrous	1.5–4.0 cm; branching; with scattered retrorse hairs	3.0–12 cm; branching; with densely short crisped hairs
Leaf	usually at base, sometimes scattered in stem; all leaves elliptic to spathulate; upper lamina surface hirsute, lower lamina surface glabrous; margin entire	scattered in stem; all leaves ovate to lanceolate; upper lamina surface hirsute, lower lamina surface glabrous; margin entire	scattered in stem; lowermost leaves orbicular, the rest oblong-spathulate; both lamina surface with short crisped hairs; margin crenately lobed
Stipule	undivided, linear, entire	undivided, linear, entire	pinnatipartite, oblong-spathulate, crenately lobed
Peduncule	up to 1.5 cm long	1.0–3.0 cm long	1.5–5.5 cm long
Bracteole	at the upper third part of the peduncle	at the upper quarter part of the peduncle	just below the flower
Sepal	glabrous, 2.0 mm long	glabrous, 3 mm long	7.0 mm long, crisped hairs at margin
Sepal appendice	glabrous, obtuse, 0.5–0.8 mm long	glabrous, acute, 1.0 mm long	crisped hairs at margin, obtuse, 2.0 mm long
Petal	up to 4.0 mm long, golden-yellow, tip of lowest petal emarginate	up to 7.5 mm long, golden-yellow, tip of lowest petal emarginate	up to 12 mm long, creamy-white to yellow, with a yellow centre, tip of lowest petal not emarginate
Spur	yellow, without purple spots	yellow, with purple spots	yellow, without purple spots

Additionally, the present study showed that Inter simple sequence repeat (ISSR) marker system is very efficient for identification of genetic variations at species level in *Viola*. Dendogram analysis based on ISSR band profiles sharply determined differentiation among species in that genus and it provided a simply molecular marker system for accession of a new *Viola* species. The four ISSR primers used in this study might simplify the comprehension of genetic diversity of close taxa in *Viola*, and they could be useful for further taxonomical research in that genus.

ISSR marker system being efficient, cheap and fast technique is often used for identifying genetic relationships (and/or distances) among individuals, populations, characterization of different animal or plant taxa. This marker system is based on variation of the regions among microsatellites. ISSR PCR has been utilized for genetic stability determination (Kaya & Souza 2017), phylogenetic analyses and cultivar identification (Kaya 2015), evaluation of hybridization (Jayavalli *et al.* 2011), detection of somaclonal variation (Bello-Bello *et al.* 2014) and analysis of genetic fingerprinting (Bornet & Branchard 2001) in many species.

Usage of both morphological and molecular characterizations are very efficient and reliable techniques for introduction of new plant species to plant genetic resources, and they are also important markers for determination of different genotypes or populations, formation and conservation of germplasm collections. Additionally, these techniques are useful for breeding programmes, utilization and evaluation of genetic resources (Franco *et al.* 2001, Rout & Mohapatra 2006, Tripathi *et al.* 2013).

This multipurpose marker technique is a valuable aid for researchers working on different areas such as conservation of biodiversity and systematic biology. The present study aimed to identify a new *Viola* species compared with two different *Viola* species (*V. dirimliensis* and *V. kitaibeliana*) using ISSR marker system and morphological characterisation.

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