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ARTICLE

Contemporary interspecific hybridization between *Dracocephalum kotschyi* and *Dracocephalum oligadenium* (Lamiaceae): Evidence from morphological, anatomical and molecular data

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ABSTRACT *Dracocephalum* is the second largest genus in the family Lamiaceae with about 186 species. These species are native in temperate regions of the Northern Hemisphere and occur in the territory of the extra-tropical Asia and Europe. Eight *Dracocephalum* species reported in Iran; these are mainly growing in the northern and central parts of the country belonging to the Irano-Turanian phytogeographical region. *Dracocephalum kotschyi* is an important medicinal plant .in the country. At the same time, taxonomic position of *Dracocephalum oligadenium* is a challenging issue. In this work morphological, anatomical and Inter Simple Sequence Repeat (ISSR) markers were used to identify these species in Iran. MDS plot based on morphological and anatomical characters, furthermore, PCoA and MST plot based on ISSR data of species revealed hybridization between *D. oligadenium* and *D. kotschyi*.
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Introduction

The genus *Dracocephalum* L. also named dragonhead (Lamiaceae) contains about 186 species (Budantsev 1987, 1993; Kadereit 2004). *Dracocephalum* species are mostly perennial, rarely annual herbs growing in alpine and semi-dry regions. They grow mainly in temperate Asia, with a few species occurring in Europe and only one in North America (Brach and Song 2006). These species have medicinal values and are used in anti-hyperlipidemic, analgesic, antimicrobial, antioxidant, anticancer treatments (Sajjadi et al. 1998; Jahaniani et al. 2005; Sonboli et al. 2008; Zeng et al. 2010).

Budantsev (1987) divided *Dracocephalum* genus into three subgenera namely, *Dracocephalum* L., *Fedtschenkiella* (Kudr.) Schischk and *Ruyschiana* (Mill.) Briq. The subgenus *Dracocephalum* includes seven sections, the members of which have glabrous anthers and stamens.

Eight *Dracocephalum* species were reported in Flora Iranica (Rechinger 1982), while Jamzad (2012) reported 10 species in Flora of Iran. Five of these species are endemic to Iran.

The occurrence of *D. oligadenium* Bornm. & Gauba in Iran is in dispute. Esfandiari (1985) reported the occurrence of both *D. kotschyi* Boiss. and *D. oligadenium* in the country, while Jamzad (2012) considered these two as synonyms and reported only the occurrence of *D. kotschyi*.

During our extensive field collection of *Dracocephalum* species in Iran, based on morphological features we encountered the presence of both *Dracocephalum* species. Therefore, the present study has been performed to differentiate these two presumed species by multiple approaches using morphological, anatomical and molecular data. Moreover, we found plants with intermediate morphological characters. Therefore, we also tried to reveal the hybrid nature of these plants by using multiple data sets.

Materials and methods

Plant materials

Plant materials (70 plant specimens) were collected from 7 geographical populations and used for morphological, anatomical, and ISSR molecular studies (Table 1). The voucher specimens are deposited in Herbarium of Shahid Beheshti University (HSBU; Tehran, Iran).

Table 1. Geographical locations of the studied populations.

No	Province	Locality	Number of samples	Altitude (m)	Longitude	Latitude
1	Mazandran	Rineh	10	2026	35 52	52 10
2	Tehran	Fasham	10	2217	35 57	21 33
3	Qazvin	Evan	10	1796	36 29	50 26
4	Mazandran	Noor	10	2043	36 12	51 48
5	Mazandran	Namarestagh	10	2370	36 03	52 03
6	Qazvin	Niroogah	10	1318	36 17	50 01
7	Gilan	Roodbar	10	1473	36 48	49 22

Morphological and anatomical studies

Altogether, 20 morphological characters and 26 anatomical characters were studied in collected plants (Table 2 and 3).

For anatomical studies, embedded materials were prepared as follows: the adult plants samples (leaves and stems) were excised and immediately fixed for 48 to 72 h in a mixture of formalin:acetic acid:ethanol (90%) of 5%:5%:50%, respectively, than stored at 4 °C until sectioning. Samples were dehydrated in a graded ethanol series and embedded. After preparation of free transverse hand sections of the lamina and stem, samples were washed with distilled water and placed in 5% sodium hypochlorite solution for 20 min for clearing then gently rinsed with distilled water. The sections were stained with aqueous solution of methylene blue (1%) and carmine and mounted on the slides using Canada balsam (Jensen 1962). Thin cut sections were studied under a microscope fitted with digital camera. Anatomical characters of stem and leaf were summarized in Table 3.

Table 2. Morphological characteristics in the studied populations.

No.	Characters
1	Habitat form
2	Margin of stem leaves
3	Plant height (cm)
4	Length of basal leaf (mm)
5	Width of basal leaf (mm)
6	Length of petiole in basal leaf (mm)
7	Length of stem leaf (mm)
8	Width of stem leaf (mm)
9	Length of petiole in stem leaf (mm)
10	Length of inflorescence leaf (mm)
11	Width of inflorescence leaf (mm)
12	Length of petiole in inflorescence leaf (mm)
13	Size of inflorescence leaf arista (mm)
14	Length of bracteole (mm)
15	Width of bracteole (mm)
16	Size of bract arista (mm)
17	Length of calyx (mm)
18	Length of corolla (mm)
19	Width of calyx (mm)
20	Size of tooth in calyx (mm)

Morphological and anatomical characters were first standardized (Mean = 0; Variance = 1) and used to establish Euclidean distance among pairs of taxa. For grouping of the plant specimens Multidimensional Scaling (MDS) and Principal Components Analysis (PCA) were used (Podani 2000). PAST version 2.17 (Hammer et al. 2012) was used for multivariate statistical analyses.

DNA extraction and ISSR assay

Fresh leaves were collected in each of the studied populations and dried in silica gel powder. The genomic DNA was extracted using cetyltrimethylammonium bromide (CTAB)-activated charcoal protocol (Sheidai et al. 2014). The extraction procedure was based on activated charcoal and polyvinylpyrrolidone (PVP) for binding of polyphenolics during extraction and on mild extraction and precipitation conditions promoting high-molecular weight DNA isolation without interfering contaminants.

Ten ISSR primers were used: (AGC) 5GT, (CA) 7GT, (AGC) 5GG, UBC810, (CA) 7AT, (GA) 9C, UBC807, UBC811, (GA) 9A and (GT) 7CA. These were commercialized by University of British Columbia (UBC).

Polymerase chain reactions (PCR) were performed in 25 µl volumes containing 10 mM Tris-HCl buffer (pH 8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Bioron, Germany), 0.2 µM of a single primer, 20 ng genomic DNA and 3 U of Taq DNA polymerase (Bioron, Germany).

The amplification reactions were performed in a thermal cycler (Techne, Germany) with the following program: 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 sec, 57 °C for 1 min, and 72 °C for 1 min, followed by one final extension at 72 °C for 7 min. The amplification products were visualized by running on a 2% agarose gel, followed by ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

ISSR bands obtained (results not shown) were coded as binary characters (presence = 1, absence = 0). Grouping was done by Neighbor Joining (NJ) clustering, Neighbor

Table 3. Anatomical characteristics in the studied populations.

No.	Characters
1	Thickness of epidermis in stem (µm)
2	Thickness of collenchymas in stem (µm)
3	Thickness of parenchyma in stem (µm)
4	Thickness of sclerenchyma in stem (µm)
5	Thickness of upper phloem in stem (µm)
6	Thickness of xylem in stem (µm)
7	Thickness of lower phloem in stem (µm)
8	Thickness of pith in stem (µm)
9	Length of transects in stem (µm)
10	Width of transects in stem (µm)
11	Length of simple hair in stem (µm)
12	Length of glandular hair in stem (µm)
13	Number of layers of collenchymas in stem
14	Number of layers of parenchyma in stem
15	Number of layers of xylem in stem (µm)
16	Thickness of upper epidermis in leaf (µm)
17	Thickness of collenchymas in leaf (µm)
18	Thickness of parenchyma in leaf (µm)
19	Thickness of upper phloem in leaf (µm)
20	Thickness of xylem in leaf (µm)
21	Thickness of simple hair in leaf (µm)
22	Thickness of glandular hair in leaf (µm)
23	Number of layers of collenchymas in leaf
24	Number of layers of parenchyma in leaf
25	Number of layers of xylem in leaf
26	Thickness of lower phloem in leaf (µm)

Net of ordination as well as PCA. They were performed after 100 times bootstrapping/permutations (Freeland et al. 2011; Huson and Bryant 2006).

Moreover, minimum spanning tree (MST) was performed to illustrate genetic affinity of the presumed hybrid plants. Data analyses were performed by using PAST ver. 2.17 (Hammer et al. 2012).

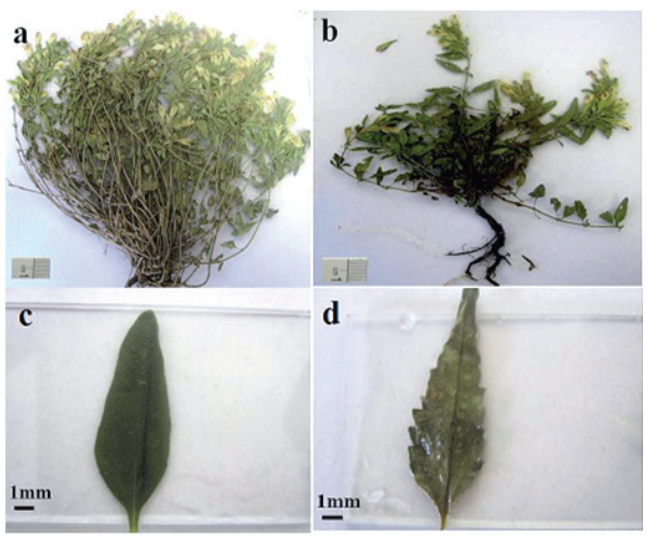

Figure 1. Habitat form and margin of stem leaves in *D. oligadenium* and *D. kotschyi*.

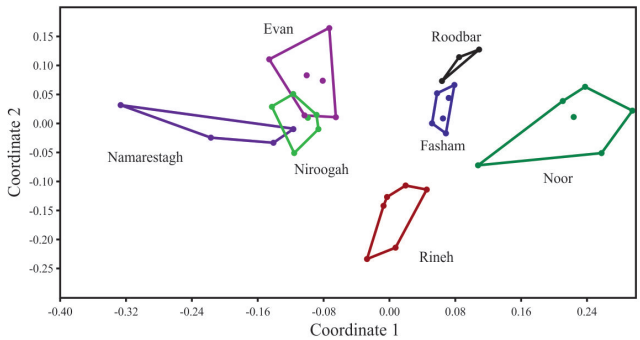

Figure 2. MDS plot of populations based on morphological characters.

Results

Morphometry

Detailed morphological investigation of the collected *D. oligadenium* (Evan, Namarestagh and Niroogah populations) and *D. kotschyi* samples (Fasham, Roodbar and Noor populations) revealed that these species mainly differ in habitat form and margin of stem leaves (Fig. 1 and Table 4). *D. oligadenium* has cartridges habitat form, while *D. kotschyi* is cartridges-cushions. Similarly, the stem leaves in the first is teethed, while they are smooth in the second one. Moreover, the plant specimens in these two species were differentiated by MDS plot based on all the studied morphological features (Fig. 2; Table 5).

We collected plants with intermediate characters in Rhine population (10 plants). They had habitat form of *D. oligadenium* and the leaf margin of *D. kotschyi*. MDS plot (Fig. 2) based on morphological characters separated *D. oligadenium* populations (Evan, Nomarestaq and Niroogah) from *D. kotschyi* populations (Fasham, Roodbar and Noor), while plants of Rineh population were placed in an intermediate position between the two species and may be considered as potential hybrids.

Table 4. Results of qualitative morphological traits in populations studies.

Species	Habitat form	Margin of stem leaves
D. oligadenium	Cartridges	Teeth of the leaves very deep
D. kotschyi	Cartridges-cushions	Teeth of the leaves not very deep

Table 5. Results of quantitative morphological traits in populations studies.

Characters	Populations						
	Rineh	Fasham	Evan	Noor	Namarestagh	Niroogah	Roodbar
Plant height (cm)	18.1	20.3	24.8	20.3	21	17.3	21
Length of basal leaf (mm)	6.2	8.3	10	9.1	8.3	7.3	10.7
Width of basal leaf (mm)	4.3	5.8	6.8	5.8	5.5	4.7	8.3
Length of petiole in basal leaf (mm)	4.1	5.7	8.8	8.2	5.8	4.7	4.3
Length of stem leaf (mm)	13	18.1	19.5	17.8	13.8	17.2	17
Width of stem leaf (mm)	6.8	8	8	8	5.8	7.4	7.5
Length of petiole in stem leaf (mm)	3	3.3	3.7	5.2	2.8	3.5	2.7
Length of inflorescence leaf (mm)	9.3	13.6	11.8	15.2	12	11.8	16
Width of inflorescence leaf (mm)	4	4.3	4.8	7	4.8	4.4	6.7
Length of petiole in inflorescence leaf (mm)	2.2	2.1	2.2	2.7	4.8	2	2
Size of inflorescence leaf arista (mm)	2.6	3	3.5	4.5	2.5	3.7	2
Length of bracteole (mm)	4.5	4.9	5.7	6.1	4.8	4.3	5
Width of bracteole (mm)	1.8	1.8	1.9	3.4	4.6	2.1	2.3
Size of bract arista (mm)	2.5	3.3	3.2	4.4	3.5	3	2
Length of calyx (mm)	16.2	14.8	16.2	14.3	13	14.7	15
Length of corolla (mm)	26.8	28.4	24.7	25.8	19.5	22.8	27.3
Width of calyx (mm)	5.3	5	4.8	4.8	4.5	5.3	5
Size of tooth in calyx (mm)	6.2	6.8	5.8	6.2	5	6.7	6

Anatomy

Details of mean of anatomical characteristics in seven studied population are provided in Table 6. PCA analysis of anatomical features revealed that the first 3 PCA components comprise about 75% of total variance. PCA revealed that anatomical characters like the thickness of collenchyma, sclerenchyma, lower and upper phloem, and xylem, as well as width of transects are the most variable anatomical characters. These characters differentiate both the species studied as well as the "hybrid population" (Fig. 3).

In this study, the stems in the cross section have a square form with pronounced angles and are covered with a one-layered epidermis. Collenchyma is single-layered among the angles but 8-11 layers of collenchyma are observed below the epidermis at the angles. Phloem and xylem were regular cylinders.

The highest thickness of parenchyma (68.81 μm), sclerenchyma (33.44 μm), lower phloem (62.4 μm) and length of glandular hair (46.51 μm) in stem was observed in Rineh population. These characters are attributes that diverge the population of Rhine from other populations.

The highest length of simple hair in stem (107.14 μm) was observed in Niroogah population, while Evan population had the highest value of thickness of epidermis (29.14 μm) and upper phloem (55.18 μm). The lowest thickness of pith (539.68 μm) and xylem (101.46 μm) in stem was observed in Niroogah.

All the leaves in the sections were bifacial (dorsiventral and amphistomatic mesophyll) type and were com-

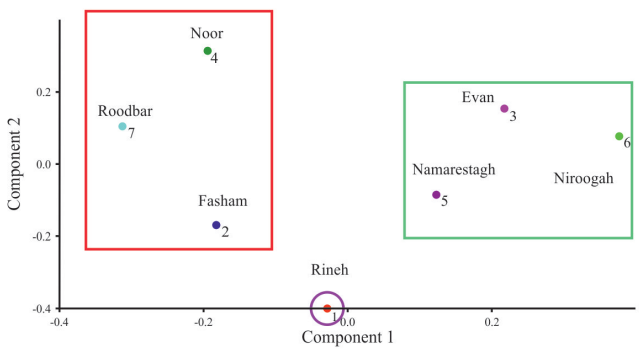

Figure 3. MDS plot of populations based on anatomical characters.

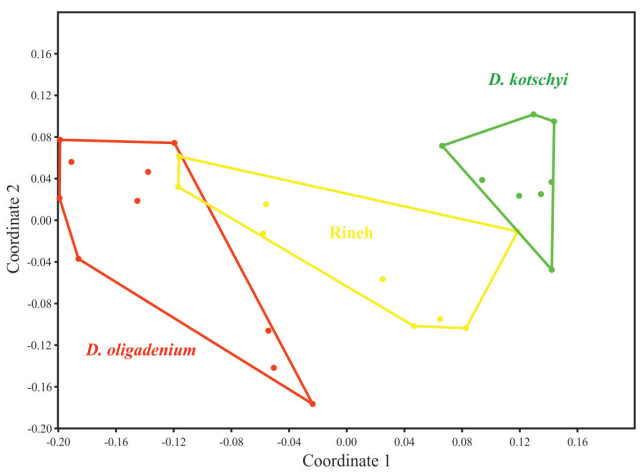

Figure 4. PCoA plot of species based on ISSR.

Table 6. Anatomical traits in the studied populations.

Characters	Populations						
	Rineh	Fasham	Evan	Noor	Namarestagh	Niroogah	Roodbar
Thickness of parenchyma in stem (µm)	68.81	50.99	60.61	49.16	63.44	61.54	43.52
Thickness of sclerenchyma in stem (µm)	33.44	15.45	30.37	20	26.34	16.66	16.66
Thickness of upper phloem in stem (µm)	39.69	28.36	55.18	35.8	38.86	46.16	30.37
Thickness of xylem in stem (µm)	146.41	152.05	157.79	165.86	162.46	101.46	214.76
Thickness of lower phloem in stem (µm)	62.4	42.81	46.86	53.68	52.68	49.16	46.86
Thickness of pith in stem (µm)	648.36	610.29	627.69	707.93	770.06	539.68	709.54
Length of transects in stem (µm)	1125.63	1111.99	1125.93	1102	1265.73	1070.68	1291.96
Width of transects in stem (µm)	1035.69	1003.26	1178.92	1154.89	1307.87	969.01	1265.76
Length of simple hair in stem (µm)	76.08	55.48	83.3	77.72	81.72	107.14	56.57
Length of glandular hair in stem (µm)	46.51	25.87	22.31	25.56	26.34	15.45	34.52
Number of layers of collenchymas in stem	8	8	11	8	11	9	10
Number of layers of parenchyma in stem	3	3	4	4	4	5	3
Number of layers of xylem in stem (µm)	12	16	20	19	20	19	18
Thickness of upper epidermis in leaf (µm)	38.33	35.25	57.14	87.41	51.11	40.41	74.51
Thickness of collenchymas in leaf (µm)	113.43	111.23	127.78	166.6	126.49	133.4	153.76
Thickness of parenchyma in leaf (µm)	234.49	140.23	143.88	193.36	247.9	225.41	179.34
Thickness of upper phloem in leaf (µm)	73.18	70.32	137.14	118.91	130.31	101.1	157.64
Thickness of xylem in leaf (µm)	185.87	157.23	208.55	199.92	206.51	159.18	165.71
Thickness of simple hair in leaf (µm)	174.07	126.32	154.39	225.12	131.55	243.31	142.97
Thickness of glandular hair in leaf (µm)	66.64	57.23	77.72	59.66	63.89	105.37	65.15
Number of layers of collenchymas in leaf	3	2	2	2	3	3	2
Number of layers of parenchyma in leaf	4	4	4	5	5	5	4
Number of layers of xylem in leaf	8	5	6	8	7	5	6
Thickness of lower phloem in leaf (µm)	174.07	126.32	154.39	225.12	131.55	243.31	142.97

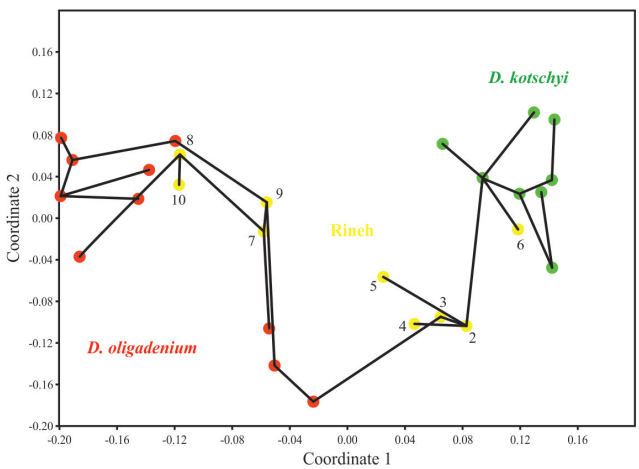

Figure 5. MST plot of species based on ISSR.

posed of one-layered epidermis. The highest thickness of the lower phloem (243.32 µm), simple (233.31 µm) and glandular (105.37 µm) hair in leaf was observed in Niroogah population and the highest thickness of the upper epidermis (87.41 µm) and collenchyma (166.6 µm) in leaf was observed in Noor population. The lowest thickness of epidermis (35.25 µm), collenchyma (111.23 µm), parenchyma (140.23 µm), xylem (157.23 µm), lower and upper phloem (70.32 and 126.32 µm, respectively), and length of glandular and simple hair (126.32 and 57.23 µm, respectively) in leaf was observed in Niroogah.

ISSR study

For ISSR studies, representatives of *D. oligadenium* and *D. kotschyi* as well as population of Rhine were analysed. PCA plot of ISSR data (Fig. 4), followed by minimum spanning tree (MST), separated *D. oligadenium* and *D. kotschyi* species from each other, while Rineh population was placed in an intermediate position. The MST plot revealed that some plants were genetically closer to *D. oligadenium* (plants 7, 8, 9 and 10 in Fig. 5); while some others were closer to *D. kotschyi* (plants 2, 3, 4, 5 and 6 in Fig. 5). This agrees with the results of the morphological and anatomical investigations.

Discussion

Species differentiation is an important taxonomic task which can be achieved through a combination of various characteristics and approaches (Sheidai et al. 2014). We could differentiate two species of *D. oligadenium* and *D. kotschyi* by using a combination of morphological, anatomical as well as molecular data. Taxonomic recognition of

these two species has been also done previously by using pollen data (Naderifar et al. 2015).

The present study also discovered hybrid plants between these two species. Natural hybridization is a widespread phenomenon in plant species and occurs in 40% of vascular plant families. The frequency of natural hybridization in plants varies among families, genera, and species pairs.

Interspecific hybridization is an important evolutionary mechanism that brings about two genomes of divergent but related species together. It can produce new genetic and phenotypic traits that can help the species ecological adaptation (Freeland et al. 2011). Interspecific hybridization occurs frequently in various plants groups but is under influence of different factors like, the genetic architecture of the species involved, the fitness of the hybrid and genotype-environment interaction (Freeland et al. 2011).

Natural interspecific hybridization occurs with high frequency in Lamiaceae family (see e.g., Gobert et al. 2002; Idowu and Oziegbe 2017; Mamadalieva et al. 2017).

The hybridization can lead to a large diversity in different characters; for example, in *Tamarix*, different species can interbreed naturally and form different hybrids with extensive range of morphological variation, depending on the degree of introgression and genetic contribution of the parental species (Ijbari et al. 2014). In case of *Helichrysum*, the hybrid plants were formed between *Helichrysum armenium* and *Helichrysum rubicundum* and showed intermediate morphological characters (Taban et al. 2015). The hybrids were formed in the hybrid zone, where the two species overlapped. The same is true in our present investigation as we collected hybrid plants in the overlapping area between *D. oligadenium* and *D. kotschyi*.

Usually, the hybrids are identified based on intermediate morphological and molecular characteristics. The same holds true for *Dracocephalum* "hybrid plants" as they were placed between the two parental taxa in MDS plot of ISSR data. Moreover, they showed intermediate anatomical features too. To our knowledge, this is the first report on the occurrence of a natural interspecific hybrid in the genus *Dracocephalum*

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