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Genetic Differentiation of two *Dracocephalum* (Lamiaceae) species and populations in Iran by Polyacrylamide Gel Electrophoresis

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ABSTRACT: Genetic diversity of 11 populations of *Dracocephalum* genus (five populations of *D. moldavica* and six populations of *D. kotschyi*) was studied by an electrophoretic pattern of total proteins of leaves and investigated to determine the range of genetic diversity. The variability of leaf storage-proteins were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Detailed protein profile analysis allows us to group the different populations and to postulate relationships among them. On the whole lot 47 reproducible bands were used for analysis and genetic diversity was estimated based on the number of different protein peptides.

Populations of *D. kotschyi* (29652, 12938 and H2200) had the highest number of bands (11) and population *D.* moldavica (909) had the lowest band (1). Electrophorogram for each population were scored and Jaccard's similarity index (JSI) was calculated. The maximum (JSI) coefficient was between populations *D. kotschyi* (12938) and *D. kotschyi* (H2200) (0.374).Clustering analysis using single linkage algorithm based on JSI Measures classified the *Dracocephalum* populations into six major groups at 4.24 metric distances. The PCOA data confirmed the results of clustering. It is concluded that leaf storage protein profiles could be useful markers in the studies of genetic diversity and classification of *Dracocephalum* species.

Keywords: Cluster analysis; *Dracocephalum*; Genetic variation; Lamiaceae; Principal Component Analysis; SDS-PAGE

INTRODUCTION

Lamiaceae family has more than 7000 species and about 280 genera and one of the largest families of plant which has a cosmopolitan distribution (Raymond *et al.,* 2004). *Dracocephalum* is a genus of about 186 species (IPNI) of flowering plants in the family Lamiaceae, native to temperate regions of the Northern Hemisphere (Nixon, 2006). They are annual or perennial herbaceous plants or sub shrubs, growing to 15 to 90 centimeters tall. In the flora of Iran, *Dracocephalum* is represented by eight species, which are mainly distributed in the northern and central parts of the country, belonging to the Irano-Turanian phytogeographical region (Rechinger, 1982). With the exception of the widespread endemic species *D. kotschyi* and the cultivated one *D. moldavica* the rest of the species (namely *D. polychaetum, D. surmandinum, D. multicaule*, *D. subcapitatum, D. aucheri* and *D. thymiflorum*) exhibit more or less highly restricted distributional patterns in Iran.

Basic chromosome numbers in all of the populations of *Dracocephalum moldavica* L. and D. kotschyi Boiss. In Iran were x = 5 (2n = 2x = 10) and x = 10 (2n = 2x = 20) (Salehi *et al.,* 2014) respectively. *D. kotschyi* Boiss. species is an endemic herbaceous plant in Iran and is known as Badrandjboie-Dennaie and Zarrin-Giah (Fattahi *et al.*, 2011; Ghahreman, 1987).

Aerial parts of *D. kotschyi* plants are sources of valuable flavonoids and essential oils (Ebrahim Sajjadi *et al.*, 1998; Gohari *et al.*, 2003; Monsef-Esfahani *et al.*, 2007; Saeidnia *et al.*, 2007) and its seeds are rich in linolenic, oleic and linoleic acids (Goli *et al.*, 2013). Recently, much attention has been paid to the *Dracocephalum* genus and its chemical constituents because of their diverse activities, such as anticancer, antioxidant, antihypoxic, and immunomodulatory activities (Zeng *et al.*, 2010). Medicinal properties and a large variety of specimens in the species increase the importance of diversity studies in this genus.

Researches showed that the different of essential oils (kind and value) in different populations of each species are variable duo to ecological and or genetic variations. For example: Biochemical studies showed that in *D. kotchyi* species citral, myrcene, β-caryophyllene and terpinyl acetate were as the main constituents from northeast mountains (Yaghmai and Tafazzoli, 1988) but (Javidnia *et al.*, 2005) reported the main components of the oil of *D. kotschyi* as α-pinene, caryophyllene oxide, terpinen- 4-ol and germacrene-D and also (Morteza-Semnani and Saeedi, 2005) reported that the major constituents of the essential oil of the *D. kotschyi* were δ-3-carene, limonene, carvacrol.

Genetic diversity is the basis for successful plant improvement and can be estimated by different methods such as morphological traits, end-use quality traits, and molecular markers (Fufa *et al.*, 2005).

Among biochemical techniques, DNA molecular markers, currently in use, are too expensive as compared to protein molecular markers, which are less expensive. The SDS- PAGE method is a suitable technique to identify varieties and was used very successfully in evaluating the genetic diversity (Sharma and Maloo, 2009). This method can also be used as a promising tool for distinguishing individuals of plant species (Camps *et al.*, 1994 and Jha & Ohri, 1996). However, a few studies indicated that cultivar identification was not possible with the SDS-PAGE method (De-Vries, 1996). The SDS-PAGE is a practical and reliable method for species identification because seed storage proteins are largely independent of environmental fluctuations (Gepts, 1989).

The assessment of the level and structure of genetic diversity of leaf storage protein in *Dracocephalum* genotypes is a requirement for plant breeding and genetic resource conservation programs. To our knowledge, no studies have yet been made in Iran on the diversity *of Dracocephalum* germplasm based on protein electrophoresis. So the present study aimed, to assess the patterns of genetic variability within and among two species of *Dracocephalum* genus in set of eleven populations using leaf storage proteins and grouping them based on these proteins as a biochemical marker.

MATERIALS AND METHODS

A. Plant materials

The materials used in this study were collected in different areas of Iran. The localities, gene bank codes and species names are shown in Table 1. Vouchers are deposited in gene bank RIFR (Research Institute of Forest and Rangelands from Iran). About 5 single plants for each population randomly sampled, and their young leaves were collected. Thus, a total number of 11 populations were characterized in this study.

Table 1. Localities of species used in the study.

Population	Gene bank code (RIFR)	Locality
Dracocephalum moldavica	909	Karaj(1)
D. moldavica	1089	Karaj(2)
D. moldavica	1613	Hamedan
D. moldavica	3429	Karaj(3)
D. moldavica	14336	Hamedan
Dracocephalum kotschyi	798	Qazvin(3)
D. *kotschyi*	18173	Isfehan
D. *kotschyi*	180	Chalus
D. *kotschyi*	12938	Qazvin(1)
D. *kotschyi*	29652	Qazvin(2)
D. *kotschyi*	H2200	Isfehan- Samirom

B. Protein extraction and electrophoresis

One or two leaves from each plant sample were randomly selected for protein extraction. The selected leaves were individually ground to a fine powder using a mortar and pestle, and 0.5g of the powder was added into an Eppendorf tube for each population with 900 µl of a protein extraction buffer. The extraction buffer contained 50mM Tris-HCl pH 8.0, 2mM EDTA, 1mM PMSF, 2% β-mercaptoethanol, 1% Triton X-100 and 20mM $MgCl_2$ (Laemmli, 1970). The Eppendorf tubes were vortexes thoroughly using an Automatic Lab Mixer DH-10 to homogenize, and the homogenate samples were purified by centrifuging at 13000 rpm for 10 min at room temperature. The extracted crude proteins were recovered as clear supernatant, transferred into new 2 ml Eppendorf tubes and stored at 4°C for electrophoresis.

Electrophoresis was performed using a discontinuous sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) system of Laemmli (1970) with 12.5% (w/v) separating gel and 5% stacking gel (Scheibe *et al.*, 2001). A five µl of supernatant was added to 1.25 µl β-mercaptoethanol and 3.75 µL sample buffer (0.002% blue bromo phenol, 0.0625M Tris-HCl pH 6.8, 0.2% SDS, 15% Glycerol) and was loaded with the micropipette into the gel wells and run at a constant current of 90 Volts at room temperature (25°C) till the tracking dye migrated to the gel bottom. The gels were later stained in 0.1% Coomassie Brilliant Blue R-250 in methanol, acetic acid and distilled water (40:10:50 v/v) for 8-10 hours.

Next they were destained in the same solution without Coomassie Brilliant Blue R-250 with occasional shaking till the gels became clear. The resulting gels were photographed to visualize the protein band patterns. Protein molecular weight marker was #SM0431 made by Fermentas Company.

C. Data analysis

After staining and destaining the gels, The number of monomorphic and polymorphic protein bands were counted for each sample and manually scored as 1 (present) or 0 (absent). A binary genetic distance matrix was obtained by visual scoring of the bands. Only strong, reproducible and clearly distinguished bands were used in the analysis. After obtaining the genetic distance matrix, clustering was performed by single linkage algorithm. An individual pair wise similarity matrix for 11 populations was generated using a Jaccard coefficient and converted to the Euclidean distance matrix for a principal coordinate analysis (PCoA) using the JMP program (JMP, 1995). The first two resulting principal coordinate scores were plotted to assess the genetic associations of 11 populations. Cophenetic correlation coefficient between the similarity matrix and the cophenetic matrix derived from the dendrogram is calculated.

RESULTS AND DISCUSSION

Information about genetic diversity and relationships among the breeding materials has a significant impact on plant improvement.

In this study, we wanted to use the molecular similarities to evaluate the species of *Dracocephalum* populations.

Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is used because in this method samples are analyzed in a more direct manner. This method is relatively easy and many samples can be analyzed at the same time. It is also cheaper than other fingerprinting methods. Moreover, the results obtained by SDS-PAGE of whole-cell proteins can discriminate at much the same level as DNA fingerprinting, in some cases (Priest and Austin, 1993)

Protein distribution patterns in 11 populations of two species of *Dracocephalum* genus were studied and reveal qualitative and quantitative variations in terms of band number, staining intensity and molecular weight. The electrophorogram showing proteins banding pattern of different populations are given in Fig.1.

On the basis of the relative mobility of leaf proteins on the gel, 47 polymorphic, strong, reproducible and clearly distinguished bands of leaf storage protein in 11 populations collected in different areas of Iran were detected, which were used for examining the genetic diversity. The results from comparison with standard molecular weight marker (Fermentas #SM0431) reveal that all studied populations of *Dracocephalum* contain some bands in range of 15 to 68 KDa (Fig.1). The range of resolution factor (Rf) was between 0.011 to 0.978 that indicated high polymorphism among the populations. Although the number of bands in different populations were comparable but the density of polypeptides in some bands were clearly different in some populations. Detailed protein profile analysis allows us to group the different populations and to postulate relationships among them.

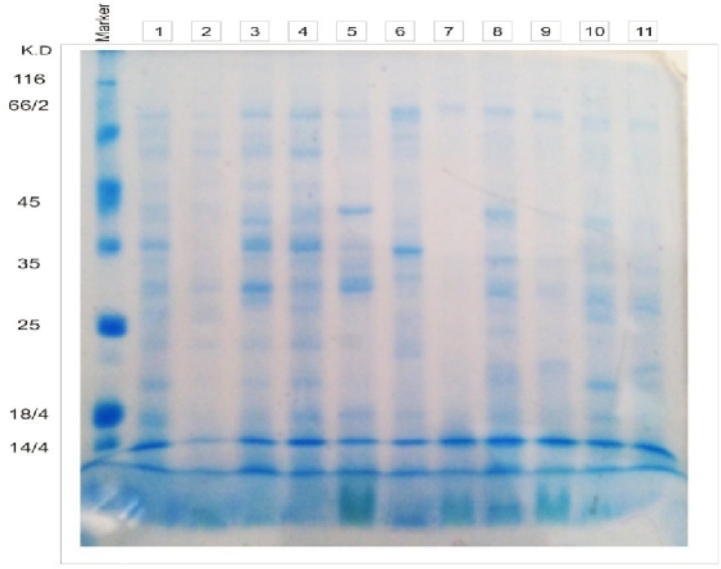

Fig. 1. Leaf storage protein profiles in 11 populations of *Dracocephalum* genus. M: molecular weight marker (14.4-116 KDa: Fermentas). 1: *D. moldavica* (1613). 2: *D. moldavica* (1089). 3*: D. moldavica* (3429). 4: *D. moldavica* (14336). 5: *D. moldavica* (909) .6: *D. kotschyi* (29652).7: *D. kotschyi* (18173).8: *D. Kotschyi* (180).9:*D. kotschyi* (12938).10: *D. kotschyi* (798). 11: *D. kotschyi* (H2200).

The total number of leaf storage protein bands observed for each population varied from 1 to 11 and averaged 8.2. The percentages of polymorphic bands over the total bands detected ranged from 2% (*D. moldavica* (909)) to 23 % (*D. kotschyi* (26652), *D. kotschyi* (12938) and *D. kotschyi* (H2200)) and averaged 17 %.

Table 2. Eigenvectors from the first sixth Principal components for 47 bands to classify 11 populations of *Dracocephalum* species.

Sixth component	Fifth component	Fourth component	third component	second component	First component	BAND
-0.01433	-0.04953	0.14424	0.15941	0.01774	0.26181	1
-0.06896	-0.15985	-0.1138	0.02434	-0.0764	-0.12809	2
-0.15158	0.12115	0.24975	-0.06139	-0.13283	-0.12613	3
0.25177	0.26211	-0.18532	-0.01765	-0.09601	0.05404	4
-0.0559	0.35597	-0.19592	0.07325	0.00564	0.02296	5
-0.05879	0.21973	-0.05826	0.15146	0.01311	0.19838	6
-0.08772	-0.16878	-0.00836	0.19296	0.03785	-0.21309	7
0.01103	-0.11775	0.04221	-0.0883	0.15902	0.18906	8
-0.18723	0.07922	0.2358	-0.16439	-0.17779	-0.03137	9
0.15388	0.05583	0.13646	-0.19478	-0.23273	0.01354	10
-0.16175	0.10477	-0.0581	0.04447	0.19142	0.1881	11
0.07737	0.25995	0.02089	0.19526	0.07655	-0.17552	12
-0.01433	-0.04953	0.14424	0.15941	0.01774	0.26181	13
0.13101	0.03485	0.17014	0.17087	0.08419	-0.21979	14
-0.02236	0.00072	0.00542	-0.25898	0.26097	0.03612	15
-0.17808	-0.13183	-0.05096	0.10074	0.03596	-0.00614	16
0.12637	-0.02672	0.1002	-0.08331	0.27901	-0.09012	17
-0.0676	-0.24667	-0.20539	-0.03303	-0.08791	-0.04516	18
0.28275	-0.04014	0.06822	0.04656	-0.08699	0.23206	19
-0.18723	0.07922	0.2358	-0.16439	-0.17779	-0.03137	20
-0.01433	-0.04953	0.14424	0.15941	0.01774	0.26181	21
-0.00149	-0.05492	0.11445	0.213	0.09612	-0.19431	22
-0.01433	-0.04953	0.14424	0.15941	0.01774	0.26181	23
0.04363	-0.19493	-0.00402	0.13604	0.0755	-0.10858	24
-0.24042	-0.24535	-0.18057	0.06979	-0.03698	-0.04598	25
0.12401	0.01638	0.21129	-0.06579	-0.1901	0.18072	26
-0.02236	0.00072	0.00542	-0.25898	0.26097	0.03612	27
-0.05369	0.32742	-0.07204	0.11574	0.0039	-0.08563	28
0.1919	-0.03656	0.12901	0.14722	0.11336	-0.15703	29
0.05751	-0.09972	0.00392	-0.1291	-0.2486	-0.10408	30
-0.02236	0.00072	0.00542	-0.25898	0.26097	0.03612	31
0.1919	-0.03656	0.12901	0.14722	0.11336	-0.15703	32
0.39368	-0.00431	-0.05272	-0.09694	-0.13445	0.04954	33
-0.0559	0.35597	-0.19592	0.07325	0.00564	0.02296	34
-0.18723	0.07922	0.2358	-0.16439	-0.17779	-0.03137	35
-0.14935	0.11823	-0.28861	0.04931	-0.05958	-0.02428	36
0.09902	0.03064	0.15085	-0.0192	0.24136	-0.16703	37
-0.01433	-0.04953	0.14424	0.15941	0.01774	0.26181	38
-0.14448	-0.19735	-0.1913	-0.0071	-0.08558	-0.05554	39
0.33351	-0.10279	-0.1021	-0.09999	-0.12433	0.03317	40
-0.02236	0.00072	0.00542	-0.25898	0.26097	0.03612	41
0.13101	0.03485	0.17014	0.17087	0.08419	-0.21979	42
0.18575	-0.15031	-0.18188	-0.07755	-0.164	-0.00448	43
-0.15158	0.12115	0.24975	-0.06139	-0.13283	-0.12613	44
-0.14341	-0.13518	0.06952	0.1939	0.04003	0.19056	45
-0.02236	0.00072	0.00542	-0.25898	0.26097	0.03612	46
-0.09025	-0.09985	0.112	-0.01567	-0.09683	-0.15747	47
3.8346	5.0213	5.7329	6.3125	7.8201	9.081	Eigen Value
8.1586	10.6836	12.1976	13.4308	16.6386	19.3213	Percentage of Variance
80.4304	72.2718	61.5882	49.3906	35.9599	19.3213	Cum Percentage of variance

Jaccard similarity coefficient based on total leaf proteins between the different populations has been presented in (Table 3). The highest similarity coefficient was 0.375 between of *D. kotschyi* (12938) and (H2200) and the lowest was zero (Table 3). Cluster analysis of *Dracocephalum* leaf storage proteins was performed on the results of SDS-PAGE using the software JMP software to find out the diversity among the given populations.

After obtaining the genetic distance matrix, clustering was performed by single linkage method. According to observations, 47 Differentiated protein profiles were recognized and clustering analysis based on JSI Measures with Cophenetic correlation coefficient (r = 0.86) classified the *Dracocephalum* populations into six major groups at 4.24 metric distance which had separated two species from each other (Fig. 3). The highest metric distance was obtained between *D. moldavica* (909) and *D. moldavica* (1613) and the lowest metric distance was obtained between *D. kotschyi* (H2200) and *D. kotschyi* (12938).

The results of cluster analysis indicated that there is genetic diversity between and within populations of two species of *Dracocephalum* genus. Also Cluster analysis showed that the cytogenetic and proteins electrophoresis markers have separated distinctly the populations based on species (Salehi *et al.*, 2014).

Table 3: Similarity matrix based on Jaccard's coefficient for leaf storage protein in two species of *Dracocephalum* genus.

	1: D.M.1613	**2: D.M.1089**	**3: D.M.3429**	**4: D.M.14336**	**5: D.M.909**	**6: D.M.29652**	**7: D.K. 18173**	**8: D.K.180**	**9: D.K.12938**	**10: D.K.798**	**11: D.K.H2200**
1: D.M.1613	1										
2: D.M.1089	0.214	1									
3: D.M.3429	0.167	0.071	1								
4: D.M.14336	0.133	0.125	0.25	1							
5: D.M.909	0	0	0	0	1						
6: D.M.29652	0.118	0.053	0.063	0	0.091	1					
7: D.K. 18173	0	0	0.083	0.143	0	0.125	1				
8: D.K.180	0	0	0.071	0	0	0.111	0.067	1			
9: D.K.12938	0.056	0.176	0.133	0.176	0	0	0.125	0.053	1		
10: D.K.798	0.067	0	0	0.063	0.125	0.118	0.071	0.063	0.118	1	
11: D.K.H2200	0	0.053	0.063	0.111	0	0	0.2	0.111	0.375	0.056	1

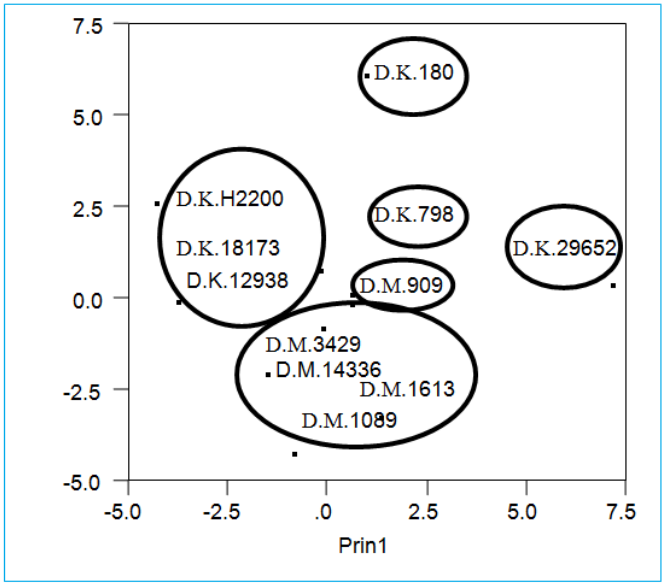

Fig. 2. Scatter plot of 11 populations for the first two principal components.

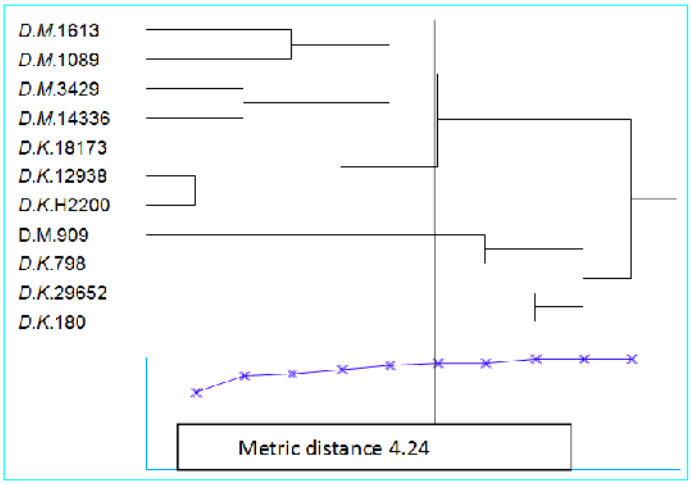

Fig. 3. Dendrogram of 11 populations of *Dracocephalum* by analyzing 47 leaf proteins bands using single linkage cluster analysis method. Cophenetic correlation $r = 0.86$.

The principal coordinate analysis (PCoA), of the protein bands shows that the first six principal coordinates account for 80.43% of total variance (Table 2). The first Component (19.32%) put emphasized on the bands 1, 7, 8, 13, 14, 21, 23, 38, 42 and 47 which had the highest coefficients of Eigen vectors, while the second component (16.6%) accentuates on the bands 10, 11, 15, 17, 27, 30, 31, 37, 41 and 46 which had the greatest role in creating variance. The third component (13.4%), put emphasized on the bands 22 and 45 and the fourth component (12.19%) accentuates on the bands 3, 9, 20, 26, 35, 36 and 44. The fifth component, which had the cum percentage of variance (72.27%) put emphasized on the bands 2, 4, 5, 6, 12, 18, 24, 25, 28, 34 and 39. finally the sixth component (8.16%) accentuates on the bands 16, 19, 29, 32, 33, 40 and 43 which had the greatest role in creating variance.

The diagram of the populations' dispersion, based on two first components showed the populations separated in six groups, which completely fits with the results obtained through the single linkage grouping analysis (Fig. 2).

CONCLUSION

In summary, the diversity within and between the species was determined by using leaf storage protein's SDS-PAGE analysis could prove useful for the rapid classification of species of *Dracocephalum* genus in relation with locality of species for the first time. The application of single linkage clustering produced two large clusters within the population, each consisting of several sub clusters.

These results suggested that protein profiles data could clearly separate different populations of two species from each other. A high correlation between protein dendrogram and geographic origin of tested genotypes was found. However, as some of the populations belong to the same species, they formed two separate groups. In the present study, SDS-PAGE analysis combined with cluster analysis confirmed the genetic similarities between some populations of the same species while it also confirmed the dissimilarities between some populations of the same species as showed by the different localities. It is therefore concluded that leaf storage protein profiles could be useful markers in cultivar and population identification, registration of new varieties, pedigree analysis, and in the studies of genetic diversity and classification of adapted cultivars, thereby improving the efficiency of *Dracocephalum* breeding programs in cultivar development.

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