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Stem anatomical study of some Iranian *Marrubium* L. species

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Abstract. *Talebi SM, Sheidai M, Ariyanejad F, Matsyura A. 2019. Stem anatomical study of some Iranian* Marrubium *L. species. Biodiversitas 20: 2589-2595. Marrubium* is one of problematic genera of Lamiaceae family which distributed in different parts of Iran with 11 species. These species have been used in folk medicine for treatment of different disorders. In the present study, we used stem anatomical characteristics of six *Marrubium* species for taxonomical treatment of the genus and delimitation of these species. Based on the geographical distribution, one to three populations were selected from each species. Stem hand transverses were double-stained and studied using Light Microscopy. In total, sixteen qualitative and quantitative anatomical traits were studied. Anatomical data were analyzed using MVSP and SPSS software. Stems in transverse section were quadrangle, with a continuous ring of vascular tissue. The anatomical characteristics varied among the species, while the ANOVA test revealed significant variations for some of them. The studied species and their populations were divided into two groups in the UPGMA dendrogram of anatomical data. Populations of each species did not cluster together and were scattered in these groups, except those for *M. cuneatum*, which were clustered in only one group. Furthermore, these groups were clearly observed in PCA plot. According to CA-joined plot, each group or population had distinct anatomical feature (s) which was useful in identification of them. In some cases, the clustering pattern agreed with previous molecular and morphological studies, while in several times populations clustering did not agree with traditional classification of species as Flora of Iran and Flora Iranica. It seems that some infraspecific ranks must be redefined.

Keywords: *Marrubium*, stem, anatomy, population, infraspecific variations

INTRODUCTION

The genus *Marrubium* L. (Lamiaceae–Lamioideae) is comprised of about 49 species (Ahvazi et al. 2016). The genus includes annual and perennial herbs. Although its taxa are mainly distributed in the Irano-Turanian as well as Mediterranean phytogeographic regions, some species are naturalized in America and Australia (Akgül et al. 2008). Some species of the genus like *M. vulgare* and *M. anisodon* are being used since ancient as a medicinal plant in the treatment of inflammation, edema, ear pain, appetizer, dyspepsia, high blood pressure, cardiac pains, spasm, flatulence, dyspepsia, and women infertility (Naghibi et al. 2005).

According to Flora Iranica (Rechinger 1982) and Flora of Iran (Jamzad 2012), *Marrubium* consists of 11 species in Iran, from them only one is endemic (*M. procerum* Bunge). Most *Marrubium* species are distributed in steppes, arid and semiarid areas of Iran (Halvorson 2003; Grassia et al. 2006). There are many discussions about infrageneric classification of the genus. Bentham (1834) revised this genus and divided *Marrubium* into two sections, namely *Lagopsis* and *Marrubium*. Boissier (1879) and Briquet (1896) divided the genus into two and three sections, respectively. However, in some countries such as Iran and Turkey, the *Marrubium* species were not classified into sections (Cullen 1982; Rechinger 1982; Jamzad 2012).

Radford et al. (1974) have suggested that anatomical characteristics have been used in taxonomical investigations for more than hundred years. Solereder (1908) summarized a lot of data about anatomical traits of some dicotyledonous families such as Crassulaceae, Dipterocarpaceae, and Myrsinaceae. Moreover, Metcalfe and Chalk (1950) gave a synthesis of previous works and their own investigations of several families.

Almeida et al. (2009) have suggested the phylogenetic use of anatomical characteristics in the delimitation of different genera. Furthermore, anatomical traits have been studied in descriptive works (Braga 1977; Benzing 2000), using an ecophysiological approach (Scarano et al. 2002), or used as a tool for taxonomic delimitations of the subgenera (Aoyama and Sajo 2003) and also for species of the genus (Proença and Sajo 2004). In addition, stem anatomical variables have great of taxonomical importance. For example, Terrazas and Arias (2003) produced a complete review about the stem anatomy and they emphasized the great relevance of anatomical features for taxonomy and evolution study.

There are few works on the anatomical study of *Marrubium* species, and most of them are descriptive study of some species such as *M. vulgare* (Moreno–Jimenez et al. 2006) and *M. anisodon* C. Koch (Talebi et al. 2019). The current investigation, describes the stem anatomical structure of six *Marrubium* species (including fourteen populations) with the aim of providing additional variables to support the taxonomic boundaries of the species, and also improvement of infrageneric classification of the genus.

MATERIALS AND METHODS

Plant materials

We studied stem anatomical characteristics of fourteen populations of six *Marrubium* species from Iran (Table 1, Figure 1). Based on the distribution range, one to three populations of each species were investigated. Samples were identified according to the descriptions provided in different sources such as Flora of Iran (Jamzad 2012) and Flora Iranica (Rechinger 1982). A voucher sample for each studied plant was deposited in the Herbarium of Arak University (AUH).

Preparation method

Samples from the middle region of adult stems were fixed in the solution of FAA (Formaldehyde Acetic acid Alcohol) 50% for 48 h and then transferred and stored in 50% alcohol (Johansen 1940). Transverse hand sections were obtained using a razor blade, bleached with sodium hypochlorite and stained with Carmine and Green Methyl (Bukatsch 1972). The thin slides were mounted in 50% glycerin and observed with an Olympus CH2 Light Microscope (40X and 100X). We observed three samples of each population.

In total, sixteen qualitative and quantitative anatomical variables were evaluated. According to Metcalfe and Chalk (1950), the studied characteristics were: epidermal cell shape, length, width and length/width ratio, parenchyma cell length, width and length/width ratio, phloem tissue width, xylem cell diameter, pith cell length, width and length/width ratio, cortical collenchyma cell length, width and length/width ratio and epidermal cell layer number.

Statistical analysis

Quantitative anatomical variables were subjected to one-way analysis of variance (ANOVA) test to determine if significant variations existed among the species and their populations for each trait measured. Mean and standard deviations of features were calculated. The mentioned analyses were performed using SPSS ver. 15. Cluster analysis was carried out based on all of the studied characteristics using Unweighted Paired Group Method with Arithmetic Mean (UPGMA), Principal Coordinate Ordination (PCO (, Principal Coordinate Analysis (PCA) and Correspondence Analysis (C.A-Joined (in Multivariate Statistical Package ver. 2 (MVSP) software (Podani 2000).

Figure 1. Distribution map of the studied species and their populations in Iran

Table 1. Localities address of the studied species and their populations

Code	Species	Localities	Voucher number
A1	*M. anisodon* C. Koch.	Markazi province, Arak, Sefidkhani mountain, 1950m.	AUH8701
A2	*M. anisodon* C. Koch.	Mazandaran province, Lasem, Polor, 2500m.	AUH8702
A3	*M. anisodon* C. Koch.	Khorasan Razavi province, Nyshabur, 1400m.	AUH8703
C1	*M. cuneatum* Russel.	Kerman province, Rabor, 1000m.	AUH8704
C2	*M. cuneatum* Russel.	Golestan province, Gorgan, 500m.	AUH8705
C3	*M. cuneatum* Russel.	Qazvin Province, Qazvin, 2100m.	AUH8706
CR1	*M. crassidens* Boiss.	Hamadan, Hamekasi, 2100m.	AUH8707
CR2	*M. crassidens* Boiss.	West Azerbaijan, 60 km Salmas to Urmia, 1700m.	AUH8708
D	*M. duabense* Murata	South Khorasan, Birjand, 1430m.	AUH8709
P1	*M. parviflorum* Fisch. & Mey.	Qom province, Salafchegan, Velashjerd, 1850m.	AUH87010
P2	*M. parviflorum* Fisch. & Mey.	Qom province, Salafchegan, Zavareh, 1400m.	AUH87011
P3	*M. parviflorum* Fisch. & Mey.	Tehran province, Lavasan, Fasham, 2000m.	AUH87012
V1	*M. vulgare* L.	Qom province, Salafchegan, Imamzadeh, 1850m.	AUH87013
V2	*M. vulgare* L.	Markazi province, Khomein, Varcheh, 1900m.	AUH87014

RESULTS AND DISCUSSION

Results

In all of studied species and their populations, the stem in the transverse section was quadrangle. There was a continuous ring of 2-3-layered epidermis tissue in the stem. At the angle of stem, collenchyma cells were below the epidermis, the layer number of these cells varied among the populations. Cortex was composed of parenchyma cells, which had different shape and size. Xylem tissue formed a continuous cylinder around the pith cells and it is traversed by narrow rays. Phloem tissue made a continuous narrow ring around the xylem. The pith was composed of different-sized parenchyma cells.

The studied anatomical characteristics of the studied species and their populations were presented in Table 2. Both qualitative and quantitative anatomical characteristics varied among the studied populations.

The epidermis cell shape varied among the studied populations. In most of them, it was elliptic, while in some populations was circular such as populations CR1, CR2, P1, P2. Moreover, the shape of cortical parenchyma cells was observed as polygonal or circular. The numbers of both types were nearly equal.

The largest (0.3 µm) and smallest (0.14 µm) epidermis cells were reported from populations A1 and P1, respectively. In addition, the widest (0.17 µm) and narrowest (0.10 µm) epidermis cells existed in populations A3, and C1 and C2, respectively. Population A3 had the largest collenchyma cells, while population A1 had the smallest one. We found largest cortical parenchyma cells in populations P3 and A2, but both populations of CR had the smallest cortical cells. We had broadest phloem tissue in populations A1 and V1; however, populations D and CR1 had narrowest phloem tissue. Widest xylem tissue was registered in populations C2, C3, and P1, while narrowest xylem tissue was observed in populations CR2 and V2.

ANOVA test revealed significant difference ($P < 0.05$) for some of the studied quantitative anatomical feature such as: epidermal layer number, collenchyma cell length, collenchyma cell width, collenchyma cell length/width ratio, and phloem tissue width (Table 3).

The studied species and their populations clustered separately in the UPGMA tree of anatomical characteristics (Figure 2). Moreover, PCA and PCO plots (Figures 3, 4) produced similar results; therefore species arrangement in the tree was discussed here. This tree had two clades; each of them was composed of seven populations. In clade A, two branches existed; branch C had two groups. Populations V1 and P2 were in the large group and the small group had population CR1. Moreover, branch D was divided into two groups. Population D, was in the small group, while the large group has composed of two sub-groups. One sub-group has been composed of two populations P1 and CR2, but another sub-group had population A2. Clade B had two branches; branch E was composed of population C1, however, branch F was large and was composed of two groups. Each group was three-membered. Group G was divided into two sub-groups, population C3 placed in a sub-group and populations P3 and A3 were clustered closely in a sub-group. This condition holds true for group H and we had two sub-groups in it. Populations A1 and V2 were clustered as a sub-group, in addition, population of C2 existed in the other sub-group.

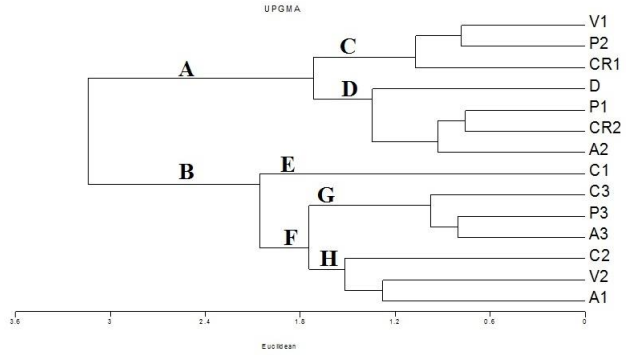

Figure 2. UPGMA dendrogram of six species and their populations according to the anatomical characteristics

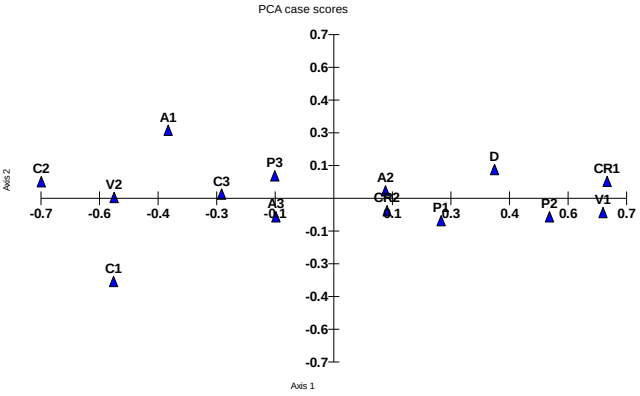

Figure 3. PCA plot of six species and populations based on the anatomical variables

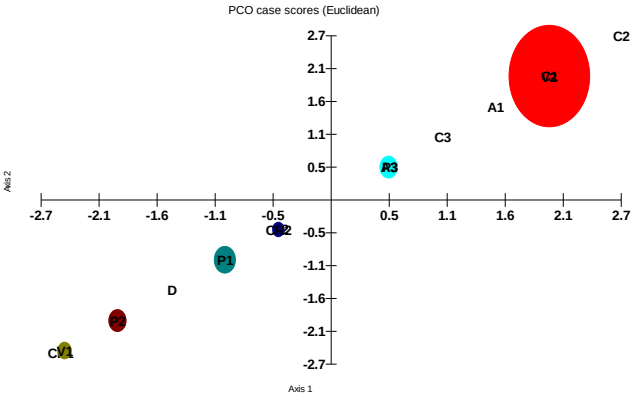

Figure 4. PCO plot of six species and their populations

Table 2. Selected anatomical characteristics of the studied species and their populations (all values are in μm)

Taxa	Epidermis cell length	Epidermis cell width	Epidermal layer number	Epidermis cell shape	Collenchyma cell length	Collenchyma cell width	Parenchyma cell length	Parenchyma cell width	Parenchyma cell shape	Phloem tissue length	Phloem tissue width	Xylem tissue length	Xylem tissue width
A1	0.30±0.12	0.12±0.06	1.50±0.70	Elliptic	0.12±0.04	0.05±0.007	0.40±0.09	0.22±0.02	Polygonal	0.16±0.00	0.13±0.02	0.12±0.007	0.10±0.02
A2	0.25±0.02	0.15±0.007	3.50±0.70	Elliptic	0.18±0.03	0.12±0.01	0.43±0.07	0.21±0.02	Circular	0.18±0.01	0.16±0.01	0.11±0.04	0.12±0.03
A3	0.23±0.02	0.17±0.02	2.50±2.12	Elliptic	0.22±0.00	0.18±0.02	0.39±0.00	0.20±0.01	Circular	0.28±0.12	0.17±0.04	0.11±0.04	0.12±0.04
C1	0.22±0.02	0.10±0.01	2.50±2.12	Elliptic	0.17±0.007	0.16±0.007	0.37±0.01	0.19±0.007	Circular	0.17±0.00	0.14±0.01	0.13±0.02	0.13±0.007
C2	0.21±0.01	0.10±0.00	3.50±2.12	Elliptic	0.17±0.007	0.16±0.007	0.33±0.04	0.20±0.01	Circular	0.17±0.007	0.14±0.007	0.13±0.03	0.14±0.01
C3	0.20±0.007	0.11±0.00	2.00±1.41	Elliptic	0.18±0.01	0.16±0.02	0.32±0.03	0.20±0.007	Circular	0.17±0.007	0.14±0.007	0.14±0.04	0.14±0.01
CR1	0.23±0.05	0.16±0.02	5.50±0.70	Circular	0.21±0.007	0.13±0.00	0.23±0.10	0.18±0.03	Circular	0.11±0.01	0.08±0.007	0.12±0.02	0.09±0.007
CR2	0.21±0.03	0.15±0.01	3.50±2.12	Circular	0.13±0.00	0.11±0.02	0.31±0.007	0.18±0.02	Circular	0.18±0.04	0.14±0.02	0.12±0.02	0.08±0.007
D1	0.21±0.03	0.12±0.01	4.50±0.70	Elliptic	0.21±0.01	0.10±0.00	0.34±0.07	0.17±0.04	Polygonal	0.11±0.01	0.08±0.01	0.12±0.007	0.11±0.02
P1	0.14±0.007	0.11±0.03	4.00±0.00	Circular	0.17±0.00	0.15±0.007	0.38±0.04	0.23±0.007	Polygonal	0.15±0.06	0.11±0.02	0.13±0.03	0.14±0.01
P2	0.19±0.04	0.13±0.04	5.00±1.41	Circular	0.15±0.02	0.13±0.007	0.33±0.04	0.17±0.04	Circular	0.11±0.03	0.09±0.03	0.10±0.03	0.09±0.03
P3	0.24±0.05	0.12±0.007	2.50±0.70	Circular	0.18±0.007	0.13±0.03	0.43±0.07	0.21±0.007	Polygonal	0.15±0.04	0.12±0.03	0.12±0.007	0.13±0.00
V1	0.18±0.01	0.12±0.007	5.50±0.70	Elliptic	0.14±0.02	0.12±0.03	0.37±0.03	0.20±0.02	Polygonal	0.22±0.00	0.17±0.01	0.11±0.02	0.10±0.01
V2	0.23±0.02	0.15±0.02	1.00±0.00	Elliptic	0.16±0.007	0.11±0.00	0.39±0.00	0.21±0.04	Polygonal	0.15±0.02	0.10±0.02	0.12±0.007	0.08±0.007

Table 3. ANOVA test among quantitative anatomical variables of the studied species and populations (Abbreviations ns: not significant, * ≤ 0.05, ** ≤ 0.01)

		Sum of squares	df	Mean square	F	Sig.
Epidermis cell length	BG	0.034	13	0.003	1.237	0.348 ns
	WG	0.030	14	0.002		
	Total	0.063	27			
Epidermis cell width	BG	0.014	13	0.001	1.486	0.236 ns
	WG	0.010	14	0.001		
	Total	0.024	27			
Epidermis cell length/width ratio	BG	53.429	13	4.110	2.302	0.067 ns
	WG	25.000	14	1.786		
	Total	78.429	27			
Epidermal layer no.	BG	3.141	13	0.242	3.119	0.022*
	WG	1.085	14	0.077		
	Total	4.225	27			
Collenchyma cell length	BG	0.024	13	0.002	5.148	0.002**
	WG	0.005	14	0.000		
	Total	0.029	27			
Collenchyma cell width	BG	0.029	13	0.002	6.615	0.001**
	WG	0.005	14	0.000		
	Total	0.034	27			
Collenchyma cell length/width ratio	BG	3.194	13	0.246	6.140	0.001**
	WG	0.560	14	0.040		
	Total	3.754	27			
Parenchyma cell length	BG	0.071	13	0.005	1.685	0.172 ns
	WG	0.046	14	0.003		
	Total	0.117	27			
Parenchyma cell width	BG	0.008	13	0.001	0.734	0.708 ns
	WG	0.012	14	0.001		
	Total	0.020	27			
Parenchyma cell length/width ratio	BG	1.240	13	0.095	1.074	0.446 ns
	WG	1.244	14	0.089		
	Total	2.484	27			
Phloem tissue length	BG	0.054	13	0.004	2.257	0.072 ns
	WG	0.026	14	0.002		
	Total	0.080	27			
Phloem tissue width	BG	0.024	13	0.002	3.101	0.022*
	WG	0.008	14	0.001		
	Total	0.033	27			
Phloem tissue length/width ratio	BG	0.405	13	0.031	1.357	0.289 ns
	WG	0.321	14	0.023		
	Total	0.726	27			
Xylem tissue length	BG	0.002	13	0.000	.192	0.997 ns
	WG	0.013	14	0.001		
	Total	0.015	27			
Xylem tissue width	BG	0.011	13	0.001	1.866	0.130 ns
	WG	0.007	14	0.000		
	Total	0.018	27			
Xylem tissue length/width ratio	BG	0.967	13	0.074	1.036	0.472 ns
	WG	1.006	14	0.072		
	Total	1.973	27			

Note: BG: Between Groups, WG: Within Groups

According to CA-joined plot, each of the separated groups had specific trait (s), which was useful in identification of them (Figure 5). For example, epidermis layer number was a good characteristic for identification of populations CR1, P2, and V1 from the rest. Furthermore, population C1 had distinct characteristics such as collenchyma cell length/width ratio, epidermis cell width, and length/width ratio.

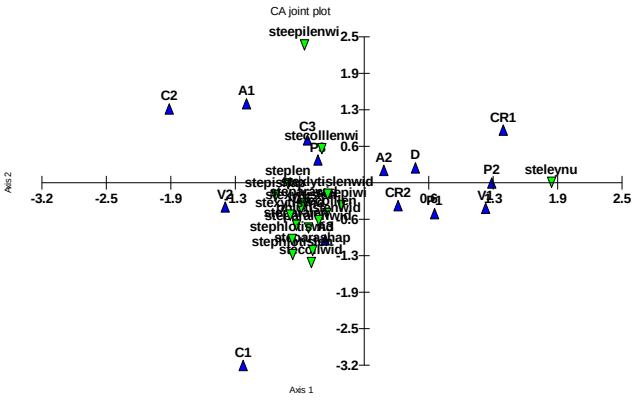

Figure 5. CA-joined plot of the anatomical variables with six species and populations (the blue symbols indicate species and their populations and the green symbols show anatomical characteristics).

Discussion

Different taxonomical patterns were proposed for the infrageneric classification of *Marrubium* species and various numbers of sections and sub-sections were definite for the genus (Bentham 1834; Boissier 1879; Briquet 1896). Moreover, taxonomic ranks of some taxa have changed in various Flora (Jamzad 2012). Therefore, in the current study, stem anatomical features were evaluated in six species of *Marrubium* in order to survey species relationship, delimitation and also comparison of these findings with previous molecular and morphological studies.

Previous researches on taxa of several dicotyledons and monocotyledons plant families (Metcalfe and Chalk 1950; Hammadi et al. 2002) have revealed the importance of anatomical characteristics on the palaeobotanical and taxonomical investigations. For example, Kantachot et al. (2007) suggested that anatomical data support morphological evidence for separating taxa at the generic level.

Moreover, we studied two to three populations from each species to examine effect of infraspecific variations on species relationship, except for *M. duabense*. Investigation of infraspecific variations has a great of importance in speciation and also species relationship at generic level (Talebi et al. 2014a, b). Previous investigations have revealed the high infraspecific morphological, phytochemical (flavonoid data), and micromorphological (calyx trichome) variations among *M. cuneatum* accessions (Kharazian and Hashemi 2017; Ahvazi et al. 2016) and also difference in leaf anatomical features in *M. anisodon* populations (Talebi et al. 2019).

The studied species and their populations were clustered separately in the UPGMA dendrogram, PCA and PCO plots of the stem anatomical characteristics. It revealed anatomical variations among the studied species and also their populations. Moreover, the ANOVA test showed significant difference for some quantitative features. We evaluated two qualitative anatomical characteristics. One of them, epidermal cell shape, varied

among the species, while was stable among the populations of the same species. The other one, cortical parenchyma cell shape, not only differed among the species, but also varied infra-specifically. Pandey (2007) has stated that traits of epidermis, vascular bundles, rays, parenchyma, and phloem cells are some of the basic anatomical characters of well established taxonomic value.

It is more important to know that all of the studied populations of the same species did not group closely. In the UPGMA dendrogram, we had two separated clades; in one clade the populations of six and in the other clade the populations of five species existed. These conditions proved high infraspecific anatomical variations. For example, we evaluated three populations from *M. anisodon*, *M. cuneatum*, and *M. parviflorum.* The populations of two species, *M. anisodon* and *M. parviflorum*, were clustered in two clades far from each other. Although, populations of *M. cuneatum* were grouped in a clade, were placed separately far from each other. Similar results were reported from both *M. crassidens* populations, which were far from in a clade.

Furthermore, in the PCA plot, axis 1 acted a cut factor and divided the studied populations into two groups. Each group was composed of seven populations, while species compositions of these groups were not similar. In the left group we had all populations of *M. cuneatum*, but the right group including M. *duabense* and both populations of *M. crassidens.*

Ariyanejad (2018) studied infraspecific genetic variations of these populations and reported high infraspecific difference among populations of *M. anisodon*, *M. cuneatum*, and *M. parviflorum.* Especially individuals of *M. parviflorum* and *M. anisodon* populations were enveloped and the boundary of the studied populations was not clear. In similar, high infraspecific genetic variations were reported from *M. cuneatum*, *M. anisodon*, *M. crassidens* and *M. vulgare* by Salehi et al. (2018).

In some cases, our anatomical findings agreed with morphological classification of the species according to Jamzad (2012) and Rechinger (1982). For example, *M. anisodon* and *M. vulgare* are very similar morphologically and the main difference of them is related to length of calyx teeth (Rechinger 1982; Jamzad 2012). In a case, one population of these species placed closely in the UPGMA dendrogram, but other populations of these species did not group together. Similar results were reported by Salehi et al. (2018). They studied genetic diversity of several populations of five *Marrubium* species using ISSR data. They found that one population of *M. vulgare* was placed among different populations of *M. anisodon,* and was clustered with one of them as a pair. Besides, *M. parviflorum* and *M. crassidens* are morphologically very similar and these species are identified based on the calyx tooth shape. In two cases, populations of these species clustered closely and made the groups, while were placed far from each other. In Ariyanejad phylogeny work (2018), these species were clustered closely in the Maximum parsimony dendrogram of ITS data by bootstrapping value of 100%.

According to Flora of Iran (Jamzad 2012), different synonyms have been definite for these species, and it reveals unstable position of these species. For example, at least five different varieties were reported for *M. vulgare*, even some authors like Trautv (1887) and Rechinger f. (1944) have believed that taxonomic rank of *M. anisodon* must be reduced as *M. vulgare* var. *arcuata* and *M. vulgare* var. *oligodon*, respectively. Meanwhile, Akgül et al. (2008) have suggested that phylogenetic analysis revealed close relations between *M. anisodon* and *M. vulgare.* Although, other populations of these species didn't cluster closely and these agreed with Salehi et al. (2018) findings of the mentioned species.

According to anatomical study, the delimitation of most species must be revised. In addition, some infraspecific ranks such as variety or subspecies must be defined for some species such as *M. parviflorum* and *M. vulgare*. Guerra and Nogueira (1990) suggested that the use of anatomical methods in taxonomic studies cannot be over emphasized. Although, no feature is absolutely immutable, some characteristics are more fixed than the others and it is on those that are less plastic that the systematic anatomist rely because they are not really affected by environmental factors. Comparative plant anatomy has been found to be reliable in taxonomy of many angiosperm taxa. Several botanists (Metcalfe and Chalk 1979; Naik and Nigrude 1981; Adedeji and Illoh 2004) have stated the taxonomic importance of anatomical characteristics, which along with other features are useful for identification and classification of plant taxa.

To conclude, we used the stem anatomical variables for taxonomical treatment of six *Marrubium* species and their populations in Iran. Most of the quantitative anatomical features did not vary significantly. However, the studied populations clustered separately in the UPGMA dendrogram and also PCA and PCO plots, and were divided into two distinct groups. Populations of the same species did not place closely and high infraspecific variations were recorded in the studied populations. Our findings agreed with previous infraspecific morphological, anatomical, molecular and phytochemical studied on these species. It seems that the some infraspecific taxonomic ranks must be redefined for some species of the genus.

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