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Genetic Diversity of *Marrubium* Species from Zagros Region (Iran), Using Inter Simple Sequence Repeat Molecular Marker

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Abstract

This study concerns the genetic diversity and taxonomic status of *Marrubium* species from central and south-west of Zagros region, Iran. It is investigated by Inter-Simple Sequence Repeat analysis. A total of 68 accessions from five *Marrubium* species were collected from their natural habitats. Molecular analysis was approved with 17 primers, of which 12 were carried out in the reaction mixture. Moreover, a data matrix was designed to estimate genetic parameters. To determine the genetic structure and taxonomic status, analysis of molecular variance, clustering analysis with *UPGMA* (Unweighted Pair Group Method Average) and the Jaccard similarity coefficient were estimated using *NT-SYS-pc* and Gene Alex software. Supplementary morphological evidences of calyx teeth features were also provided. The results of this study revealed that both sections *Marrubium*, *Microdonta*, and *Ballota aucheri* displayed a high percentage of polymorphism (PP=100%). In addition, their genetic diversity (Gst=0.99), number of effective alleles (Ne=1.53) and Shannon information index (I=0.51) showed a high percentage. Notably, all of the 12 primers produced reproducible bands. Analysis of molecular variance detected low quantities of gene variation among species (18%) from which high proportion of variation presented among populations within species (82%). Based on cluster analysis, *M. cuneatum*, *M. vulgare* and *M. anisodon* were definitely separated. Moreover, *M. crassidens* and *M. vulgare* were closely grouped. The calyx teeth features of *M. cuneatum* and *M. crassidens* revealed high variations which is consistent with molecular results. In conclusion, high genetic diversity in *Marrubium* species and accessions presents a valuable genetic resource in Zagros region, Iran.

Keywords: Genetic diversity; ISSR; Lamiaceae; *Marrubium*.

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Introduction

Marrubium L. is belonging to mint family (Lamiaceae/ Lamioideae) that is one of the important medicinal genera [4]. Its species grow as annual, perennial, herbaceous and suffruticose plants [40]. *Marrubium* species mainly distribute in Irano-Turanian and Mediterranean phyto-geographical regions [6]. This genus includes approximately 40 species throughout the world of which 11 species are reported in Iran [4, 40]. Moreover, the adaptation of these species is evident by its ability to grow in different ecological conditions especially in different kind of soils [18, 40].

As a traditional medicinal plant, *Marrubium* is known to have beneficial therapeutic properties including anti-cancer, anti-microbial, anti-hypertensive, analgesic, anti-inflammatory, expectorant effects, hyperglycemia, dyslipidemia and in the treatment of diabetes mellitus owing to its high antioxidant properties [14, 31, 43].

From a taxonomical perspective, the classification of *Marrubium* was inconsistent. This genus was divided into various sections using morphological characters. *Bentham* defined two sections for this genus [8]. *Boissier* divided *Marrubium* into two sections [9]. *Briquet* considered three sections [10] and *Knorring* defined one section and two sub-sections [22]. Moreover, *Seybold* clarified four sections [40] for 14 species (Table 1), but *Cullen*, *Akgul* and *Jamzad* did not refer to any section for this genus [4, 12, 17]. It is noted that the species limits have been disturbed by high morphological variations, hybridization, speciation and polymorphism features [4, 40].

At present, there are limited infra-genus reports of morphological studies for *Marrubium* species. *Karazian* and *Hashemi* reported high morphological variations and taxonomic status of different *Marrubium* species from Zagros region, Iran [21]. Based on micro-morphological studies, *Abu-Asab* and *Cantino* and *Akgul* et al. evaluated the taxonomy of Turkish *Marrubium* species using pollen features [1, 4]. *Ahvazi* et al. also studied the micro-morphology of trichomes and the role of environmental factors in *Marrubium* species [3].

Based on molecular markers, the Inter-Simple Sequence Repeat-Polymerase Chain Reaction (*ISSR-PCR*) procedure is a known method for identifying the DNA polymorphism in microsatellite sequences. It is noted that genetic diversity and inter and intra-specific variations and distinguishing a higher number of polymorphic fragments have been recognized using this technique [16, 25]. Remarkable evidences in different taxa and population genetics studies were proved by this method [7, 35]. This marker includes *PCR* amplifications of DNA using a single primer contained of a microsatellite sequence [5].

Nothing is known regarding the molecular systematic and genetic diversity of the genus *Marrubium*. Some molecular investigations [27, 34, 39, 45] are related to the other members of Lamioideae or Lamiaceae. These researches are mainly focused on the phylogenetic approaches. The phylogeny of Tribe Phlomideae was provided for the genera *Phlomioides* Moench and *Micromeria* Benth. using nuclear ribosomal and chloroplast sequence [27, 34, 39, 45]. Furthermore, phylogenetic relationships of the genus *Stachys* L., sect. *Eriostomum* (Hoffmanns. & Link) Dumort. in Turkey were identified via nuclear ribosomal ITS sequences [13].

Based on previous results of genetic diversity, *Song* et al. distinguished genetic diversity and population structures by *ISSR* and Sequence-Related Amplified Polymorphism (*SRAP*) molecular markers in *Salvia miltiorrhiza* Bunge. which showed high levels of genetic diversity within the populations [42]. *Kochieva* et al. characterized high polymorphism in Russian *Stachys* using *ISSR* and Random Amplified Polymorphism DNA (*RAPD*) [23, 24]. They also determined the phylogenetic position of this genus [23, 24]. Moreover, *Kharazian* et al. illustrated genetic diversity and taxonomic status of 15 Iranian *Stachys* species using *ISSR* [20]. Genetic variability of *Nepeta*

Table 1. The classification history of *Marrubium* based on taxonomic literature

Bentham (1834, 1848)	**Boissier (1879)**	**Briquet (1896)**	**Knorring (1954)**	**Seybold (1982)**
1) *Marrubium* sect. *Lagopis* Benth. 2) *M.* sect. *Marrubium* Benth.	1) *M.* sect. *Eumarrubium* Boiss. 2) *M.* sect. *Ballotoides* Boiss.	1) *M.* sect. *Ballotoides* 2) *M.* sect. *Marrubium* 3) *M.* sect. *Lagopsis*	*M.* sect. *Marrubium* with two sub-sections including 1) *M.* sub-sect. *Decendentata* Briq. 2) *M.* sub-sect. *Quinquedentata* Briq. with two groups including 1) *Stellata* Briq. 2) *Microdonta* Briq	1) *M.* sect. *Marrubium* 2) *M.* sect. *Afghanica* Seybold 3) *M.* sect. *Stellata* (Briquet) Seybold 4) *M.* sect. *Microdonta* (Briquet) Seybold

L., *Micromeria* Benth. and *Culina* D. Royen. ex L. was also investigated using *ISSR* molecular markers [2, 28, 41]. High genetic diversity in some of the Iranian *Salvia* L. species was detected using Amplified Fragment Length Polymorphism (*AFLP*) molecular markers which illustrated considerable variations within populations [38].

There are no reports in the literatures regarding molecular evidence of *Marrubium* species. It is reasonable to assume that there would be a considerable levels of variation in the case of morphological traits and high hybridization in infra-specific levels. Moreover, Zagros region is one of the genetic resource foundations to conserve biodiversity of plant taxa [20]. Consequently, there is a need for detecting the genetic diversity of *Marrubium* species in this region. Accordingly, the aims of this study are as follows: 1) assessment the taxonomic status of five *Marrubium* species from central and south-west of Zagros region using *ISSR* molecular markers, 2) comparison the morphological features such as shape of calyx teeth with the molecular data among *Marrubium* species and 3) identify the genetic diversity, population genetic structure and the genetic relations of *Marrubium* species utilizing *ISSR* markers for the first time in Iran.

Materials and Methods

Taxon Sampling

Overall, 68 accessions were collected from different habitats of central and south-west of Zagros region

Table 2. Locality of *Marrubium* and *Ballota* species from Iran

Species/accessions/herbarium No.	Locality	Height (m)	Longitude, latitude
M. cuneatum 1, 2	Chaharmahal va Bakhtiari-Soureshjan	1805-1832	32°19’N,50°40’E
M. cuneatum 5,6, 7	Chaharmahal va Bakhtiari- Rostam abad	1645-1732	32°5’N,50°32’E
*M. cuneatum*8,9	Chaharmahal va Bakhtiari- Alikouh	1638-1851	32°7'N 50°30'E
*M. cuneatum*10	Chaharmahal va Bakhtiari- Haji abad, Boldaji	1791	31°55'N 50°54'E
M. cuneatum 16,17	Chaharmahal va Bakhtiari- Farsan	1778-1683	32°15'N 50°34'E
M. cuneatum 18,19,20,21	Chaharmahal va Bakhtiari- Hirkan, Chamouran	1600-1735	32°10'N 50°26'E
M. cuneatum 29,31,32	Chaharmahal va Bakhtiari- Cheghakhor	1671	31°55'N 50°54'E
M. cuneatum 33	Chaharmahal va Bakhtiari- Bajgiran	1614	31°55'N 50°36'E
M. cuneatum 35,36	Chaharmahal va Bakhtiari- Helen forest, Firouz abad	1906-2016	31°46'N 50°42'E
M. cuneatum 50	Chaharmahal va Bakhtiari- Bare Morde, Gandomkar	1853	31°49'N 50°33'E
M. cuneatum 51,52	Chaharmahal va Bakhtiari- Lordegan, Chelegah, Kouh-e Rig	1800-2338	31°20'N 50°58'E
M. cuneatum 13,72,73	Chaharmahal va Bakhtiari- Cheliche, Emamzade Saeid	1800-1920	32°13'N 50°37'E
M. cuneatum 71	Chaharmahal va Bakhtiari- Chaleshtor	2000	32°22'N 50°47'E
M. cuneatum 37	Isfahan- 75 km from Dran, Heidari	1792	33°2'N 50°26'E
M. cuneatum 43,44,45	Isfahan- 15 km from Khansar	1665	33°03′N50°29′E
M. cuneatum 48	Isfahan- Khansar, Golestan kouh	2412	33°9'N 50°20'E
M. crassidens 3,4	Chaharmahal va Bakhtiari- Cheliche, Juneghan	1833	32°13’N,50°37’E
M. crassidens A (73), 74	Chaharmahal va Bakhtiari- Chaleshtor	2000	32°22'N 50°47'E
M. crassidens 41,42	Isfahan-Khansar, Darehbid	1689	33°4'N 50°27'E
M. crassidens 45	Isfahan- Tarar, Daran	1900	32°56'N 50°27'E
M. anisodon 16	Chaharmahal va Bakhtiari- Doabsamsami	2500-2586	32°9'N 50°16'E
M. anisodon 18, 23,25	Chaharmahal va Bakhtiari- Doabsamsami, Birakan	2500-2568	32°10'N 50°18'E
M. anisodon 20, 21	Chaharmahal va Bakhtiari- Marbore, Abbarik	2516-2525	32°25'N 50°14'E
M. anisodon 28,29	Isfahan- 15 kmm from Rozveh	1693-1749	32°47'N 50°36'E
M. anisodon 39	Kohgiloye va Boyerahmad-Toutnadeh	1350	30°53'N 51°20'E
M. anisodon 40, 59	Kohgiloye va Boyerahmad- 10 km from Sisakht	2066	30°51'N 51°29'E
M. anisodon 41	Kohgiloye va Boyerahmad- Kouhgol	2114	30°51'N 51°31'E
M. vulgare 17, 24	Chaharmahal va Bakhtiari- Doabsamsami	1768	32°9'N 50°16'E
M. vulgare 27, 38	Isfahan- toward Chadegan	1756	32°46'N 50°37'E
M. vulgare 42, 63	Kohgiloye va Boyerahmad- Kouhgol	2490	30°51'N 51°31'E
M. astracanicum 11	Chaharmahal va Bakhtiari- Kouh-e Chirou, Hoseinsanabad	2350	31°50'N 51°4'E
M. astracanicum 12,15	Chaharmahal va Bakhtiari- Kouh-e Chirou	2470-2500	31°50'N 51°4'E
M. astracanicum 22	Chaharmahal va Bakhtiari- Doabsamsami	1769	32°9'N 50°16'E
M. astracanicum 26	Chaharmahal va Bakhtiari- Marboreh, Abbarik	2555	32°25'N 50°14'E
M. astracanicum 46,47,49	Isfahan- Khansar, Golestankouh	1631-2447	33°9'N 50°20'E
*M. astracanicum*66	Kohgiloye va Boyerahmad- Kouhgol	2696	30°51'N 51°31'E
B. aucheri 64	Kohgiloye va Boyerahmad- Sisakht, kouh-e Mishi	1850	30°51'N 51°31'E
B. aucheri 65	Kohgiloye va Boyerahmad- Sisakht, Kouhgol	2696	30°51'N 51°31'E

including five *Marrubium* species with one variety and two *Ballota aucheri* Boiss. accessions (Table 2). These five species belong to *M.* sect. *Marrubium* Benth. (*M. cuneatum* Banks & Sol., *M. crassidens* Boiss. var. *brevidens* Seybold, *M. vulgare* L. and *M. anisodon* K. Koch.) and *M.* sect. *Microdonta* (Briquet) Seybold (*M. astracanicum* Jacq.). The voucher specimens were deposited in the Herbarium of Shahrekord University. It is noteworthy that these five *Marrubium* species mainly distribute in central and south-west of Zagros region.

In the case of morphological investigations, the shape of calyx teeth was studied in *Marrubium* species using a digitalized Olympus SZX-ZB12 research stereo microscope.

Due to the close taxonomic relationships between *Marrubium* and some *Ballota* species (*B. aucheri*), molecular investigations were first performed with those taxa.

DNA Extraction

Leaf samples were stored at −20°C until DNA isolation. DNA was extracted from 0.5 g fresh leaves using the method of *Khanuja* et al. with minor modifications [19]. In order to verify the quantity and quality of DNA samples, a spectrophotometer with 260–280 nm and 0.1% agarose gel with Tris–Borate–Ethylene-diaminetetra-acetic-acid (*TBE*) was applied. A total of 35 *Marrubium* accessions were selected for extraction (Table 2).

ISSR-PCR Amplification

To facilitate genomic DNA amplification, ISSR extension analysis was applied [24]. *ISSR* analysis was approved with 17 primers of which 12 were suitable to intensify (Table 3) [24]. Twelve primers were investigated in the reaction mixture; all reactions were carried out in a 25 µl volume including 1 µl of template DNA, 1 µl primer, 0.5 µl each deoxyribonudeotide triphosphates (*dNTPs*) (*Datp, Dttp, Dctp, Dgtp*), 1.25 µl $MgCl_2$, 0.4 µl of *rTaq* DNA polymerase and 16.5 µl dH_2O in a suitable 2.5 µl 10X buffer with 2 µl *DMSO*. DNA amplification was performed on an Eppendorf Mastercycler gradient 5331 thermal cycler and the amplification conditions were achieved through initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 45 seconds and elongation at 72°C for 1 minute. A final extension was planned at 72°C for 5 minutes. The amplification products were separated by 1.2% agarose gel, run at 90 V in 0.5× *TBE* and were stained with 15.2 µl of ethidium bromide with a 50-1500 bp DNA ladder as a molecular size marker [24]. The amplified genomic DNA bands were visualized and photographed under ultraviolet light (*UV*) using *GEL DOC UV Tech*.

DNA Analysis

To perform DNA analysis, all of the clear and reproducible amplified bands were chosen and scored as present (1) and absent (0). Then, a data matrix was designed to estimate genetic diversity in terms of different coefficients. Different genetic diversity parameters were applied in this research [29, 32, 44]. The additional measurement of partitioning genetic variation was obtained through analysis of molecular variance (*AMOVA*) to determine the genetic structure and genetic variability among species and populations

Table 3. Properties of ISSR markers used for *Marrubium* and *Ballota* species

Primer name	Nucleotide sequence (5'-3')
ISSR1	ACCACCACCACCACCACC
ISSR2	CTCTCTCTCTCTCTCTA
ISSR3 (UBC808)	AGAGAGAGAGAGAGAGC
ISSR4 (UBC815)	CTCTCTCTCTCTCTCTG
ISSR5	CACACACACACACAYC
ISSR6 (UBC856)	ACACACACACACACACYA
ISSR7	GAGAGAGAGAGAGAGACC
ISSR8 (UBC809)	AGAGAGAGAGAGAGAGG
ISSR9 (UBC825)	ACACACACACACACACT
ISSR10	ACACACACACACACACT
ISSR11 (UBC827)	ACACACACACACACACG
ISSR12 (UBC854)	TCTCTCTCTCTCTCTCRG
ISSR13 (UBC810)	GAGAGAGAGAGAGAGAT
ISSR14 (UBC841)	GAGAGAGAGAGAGAGAYC
ISSR15 (UBC873)	GACAGACAGACAGACA
ISSR16 (UBC855)	ACACACACACACACACYT
ISSR17 (UBC861)	ACCACCACCACCACCACC

***** Y: mixed base positions for C and T, R: mixed base positions for A and G

within the species using Gene Alex 6.5 software [33].

A dendrogram was also generated by *UPGMA* (Unweighted Pair Group Method Average) and *SAHN* clustering methods (Sequential Agglomerative Hierarchical Non-overlapping) to illustrate the taxonomic relationships. Moreover, similarity matrix based on the Jaccard's coefficient was initially estimated using *SIMQUAL* program with *NT-SYS-pc* v.2.02 software [36]. The correlation and linear regression between genetic distance and geographical distance were estimated by Gene Alex 6.5 and *SPSS* v.20 [30].

Results

The results of *ISSR* amplification showed that among the 17 primers used to detect polymorphic bands, 12 generated clear profiles and these were chosen for further studies. The fragments of 500-1500 bp were provided for five *Marrubium* species. Moreover, the 1000-1500 bp (*ISSR* 4, 6) and 600-800 bp (*ISSR* 13, 14, 15) bands illustrated the unique bands arising in all of the studied species. A total of 95 bands were identified for 35 *Marrubium* accessions (*M.* sect. *Marrubium* and *M.* sect. *Microdonta*) and two *Balota aucheri* accessions. It is revealed that all bands were polymorphic. For both sections and *B. aucheri*, the highest number of polymorphic bands was observed in *ISSR* primer no.17 (NP=12) and the lowest was in primer nos. 14 and 4 (NP=3). The mean number of polymorphic bands was 7.9. The highest polymorphic information content (PIC=0.45, 0.44) was observed in primer nos.4 and 13 and the lowest was (PIC=0.17) in primer no.12 (Table 4). The percentage of polymorphism (PP) was found to be 100% which was detected for all primers used. The highest value of marker index (MI=4.7, 3.9) was observed in primers nos. 16 and 8. The lowest value (MI=1.05) was found in primer no. 12 (Table 4).

Because of high morphological variations in shape of calyx teeth (Fig. 5), the molecular analysis was investigated separately among *M. cuneatum* accessions. In the case of *M. cuneatum* accessions, the highest number of polymorphic bands belonged to *ISSR* primer no. 16 (NP=10) and the lowest one was observed in *ISSR* primer no. 4 (NP=2) (Table 4). The mean number of polymorphic bands was 6.25. The highest PIC=0.36 was observed for primer nos.13, 16 and 8 and the lowest value (PIC=0.18) was in primer no. 12. Noticeably, the PP detected was 100% which was observed in primer nos. 9, 8, 11, 12, 13, 14, 15 and 16 (Table 4). The highest value of MI=3.6 was identified

Table 4. Molecular information for each primer used for *M.* sect. *Marrubium*, *M.* sect. *Microdonta*, *Ballota aucheri* and *M. cuneatum* accessions. N, number of loci; NP, number of fragment polymorphism; PP, percentage of polymorphism; PIC, polymorphic information content; MI, marker index

M.* sect. *Marrubium, M.* sect. *Microdonta,* and *Ballota aucheri														
Primer ISSR /character	**3**	**4**	**6**	**8**	**9**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**Sum**	**Mean**
N	11	3	6	11	6	9	6	8	3	9	11	12	95	7.9
NP	11	3	6	11	6	9	6	8	3	9	11	12	95	7.9
PP	100	100	100	100	100	100	100	100	100	100	100	100	1200	100
PIC	0.29	0.45	0.29	0.35	0.32	0.37	0.17	0.44	0.41	0.26	0.43	0.28	4.06	0.33
MI	3.2	1.3	1.7	3.9	1.9	3.3	1.05	3.5	1.2	2.3	4.7	3.4	31.4	2.6
***M. cuneatum* accessions**														
Primer ISSR /character	**3**	**4**	**6**	**8**	**9**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**Sum**	**Mean**
N	8	3	5	8	6	8	6	8	3	8	10	10	83	6.9
NP	5	2	4	8	6	8	6	8	3	8	10	7	75	6.25
PP	62	66	80	100	100	100	100	100	100	100	100	70	1078	89.8
PIC	0.24	0.26	0.24	0.36	0.29	0.32	0.18	0.36	0.32	0.28	0.36	0.26	3.74	0.28
MI	0.75	0.35	0.77	2.9	1.7	2.6	1.09	2.8	0.97	2.2	3.6	1.28	21.01	1.75
M.* sect. *Marrubium														
Primer ISSR /character	**3**	**4**	**6**	**8**	**9**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**Sum**	**Mean**
N	8	3	6	10	6	9	6	8	3	9	11	11	90	7.5
NP	8	3	6	10	6	9	6	8	3	9	11	10	89	7.4
PP	100	100	100	100	100	100	100	100	100	100	100	90	1190	99.1
PIC	0.29	0.28	0.30	0.31	0.31	0.28	0.20	0.38	0.39	0.23	0.32	0.20	3.49	0.29
MI	2.3	0.84	1.8	3.1	1.8	2.5	1.2	3.1	1.2	2.1	3.5	1.8	25.2	2.1

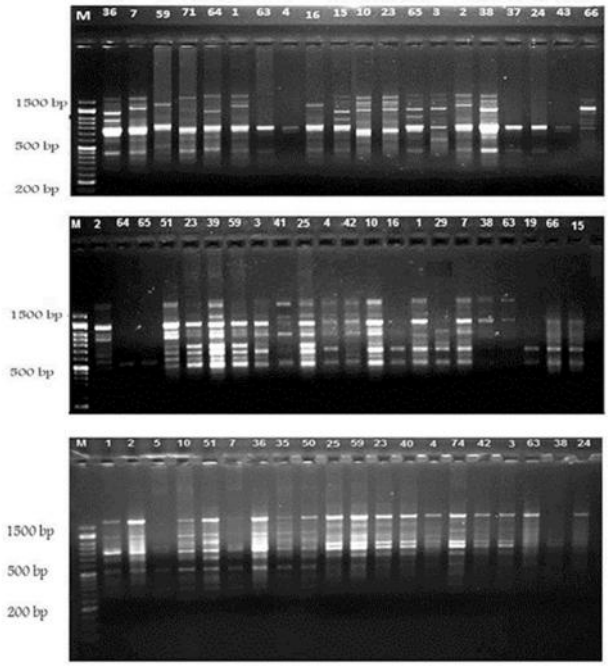

Figure 1. Banding patterns of ISSR primers with amplified loci in *Marrubium* species. *M. cuneatum*: 1, 2, 5, 7, 10, 16, 19, 29, 35, 36, 37, 43, 51, 50, 71; *M. crassidens* var. *brevidens*: 3, 4, 42; *M. vulgare*: 24, 38, 42, 63; *M. anisodon*: 3, 23, 25, 39, 41, 40, 59, 74; *M. astracanicum*: 15, 66; *B. aucheri*: 64, 65

in primer no. 16 and the lowest one (MI=0.35) was in primer no. 4 (Table 4). Noticeably, from a total number of 83 bands assessed 75 were polymorphic (Table 4).

The primer position was also estimated in *M.* sect. *Marrubium*. The mean number of polymorphic bands was 7.4 and the highest value (NP=10 and 11) belonged to primer nos. 17, 8 and 16. Both MI and PIC values were ranged from 0.84-3.5 and 0.2-0.39, respectively (Table 4). A total number of 90 bands were identified from which 89 bands were polymorphic (Table 4).

The *ISSR* banding patterns of appropriate primers are shown in Figure 1. As a result of this section, a total of 12 primers were appropriate to detect polymorphic bands in *Marrubium* species but the primer nos. 17, 13 and 16 were mainly considered to illustrate the variability information (Table 4). In *M. cuneatum*, eight primers were appropriate to detect the polymorphism and the primer nos. 8, 13 and 16 displayed the best evidence of diversity (Table 4). In *M.* sect. *Marrubium*, all primers were suitable to illustrate polymorphic bands (Table 4) but primer no. 16 was the most appropriate marker for our purposes.

The genetic diversity parameters were also estimated for the both sections of *M.* sect. *Marrubium*, *M.* sect. *Microdonta* and *Ballota* species. Overall, genetic diversity (Gst) and genetic diversity within individuals (Fis) were 0.98 and 0.01, respectively. Moreover, the Shannon information index (I) was found to be 0.51 (Table 5). The linear regression between geographical distance and genetic distance is also provided for this part (Fig. 2). The results suggest that there is little correlation between the both cases (r=0.43).

In *M. cuneatum* accessions, genetic diversity (Gst) and genetic diversity within individuals (Fis) were 0.86 and 0.14, respectively. Also, the Shannon information index (I) was found to be 0.55. Regarding the high genetic diversity, the high polymorphism in primers (PP=89.8%) was certainly expected in this case (Tables

Table 5. Inbreeding coefficient within individuals or genetic diversity within individuals (Fis or Gis), genetic diversity (Gst), expected heterozygosity (He), number of different alleles (Na), Shannon information index (I) and number of effective alleles (Ne) of *Marrubium* and *Ballota* species and *M. cuneatum* accessions

Species/accessions	Fis	Gst	He	Na	I	Ne
M. sect. *Marrubium*, *M.* sect. *Microdonta* and *B. aucheri*	0.01	0.98	0.34	1.96	0.51	1.53
M. cuneatum	0.14	0.86	0.37	2	0.55	1.60
M. sect. *Marrubium*	0.012	0.99	0.35	2	0.53	1.57

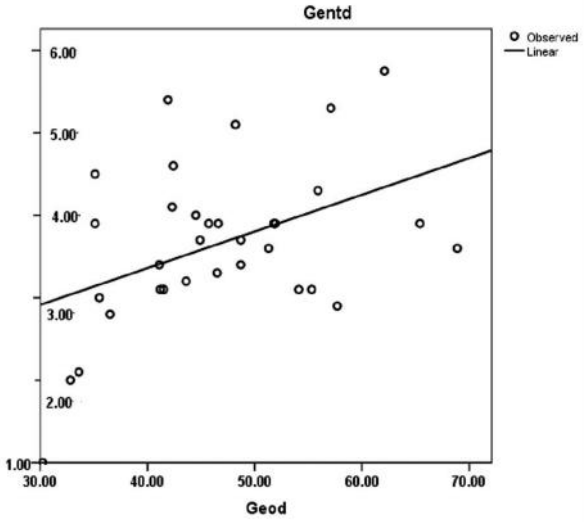

Figure 2. Linear regression between genetic distance (Gentd) and geographical distance (Geod) in the both sections *Marrubium* and *B. aucheri*

Table 6. Genetic structure in total *Marrubium* and *Ballota* species using AMOVA analysis

Source	Df	Sum of Square	Variance Component	Percentage of Variation P < 0.001
Among species	5	4.769	0.096	18%
Among population within species	30	13.509	0.450	82%
Total	35	18.278	0.546	100%

4 and 5).

In *M.* sect. *Marrubium*, evidence of genetic diversity was also estimated. It was found that, genetic diversity was high (Gst=0.99) and the Shannon information index was 0.53. Similarly, genetic diversity within individuals (Fis= 0.012) was low and the PP was found to be 99.1% (Table 5).

AMOVA analysis also demonstrated a high quantity of genetic variation presented among populations within the species (82%) and a low quantity presented among species (18%). *AMOVA* analysis also identified a high significant genetic differentiation among the populations (Table 6).

The results of cluster analysis for the both sections of *M.* sect. *Marrubium*, *M.* sect. *Microdonta* and *Ballota* species showed two groups (Fig. 3). In

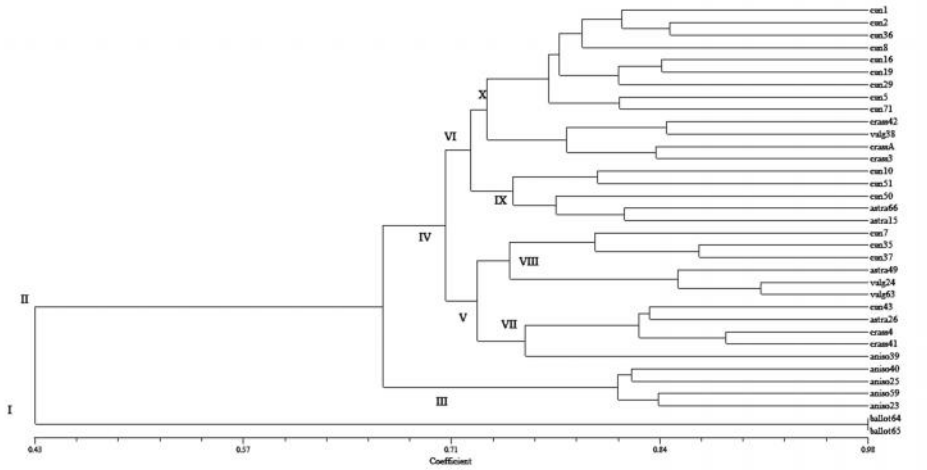

Figure 3. Dendrogram of five *Marrubium* species belonging to *M.* sec. *Marrubium* and *M.* sec. *Microdonta*, and *B. aucheri* using ISSR molecular markers. Cun: *M. cuneatum*, astra: *M. astracanicum*, vulg: *M. vulgare*, crass: *M. crassidens*, aniso, *M. anisodon*, ballot: *Ballota aucheri*

taxonomic point of view, most of the *M. anisodon*, *M. vulgare*, *M. cuneatum*, *M. crassidens* and *B. aucheri* accessions were definitely grouped and discriminated. Some of the species including *M. astracanicum*, *M. vulgare* 38 and *M. cuneatum* 43 were positioned in different taxonomic groups. Therefore, *M. astracanicum* displayed different groups with *M. cuneatum*. Moreover, *M. vulgare* clustered with *M. crassidens*.

The results of the second cluster analysis of *M. cunetaum* accessions encompassed two groups (Fig. 4). Based on these results, ten different groups were identified. Noticeably, *M. cuneatum* 29, 37, 7 and 35 are different accessions from the others. These accessions displayed different morphological features in the shape of calyx teeth. It can be concluded that these *Marrubium* accessions exhibited intra-specific variations, intermediate species and polymorphism events. These variations are provided in Figure 5 using morphological characters. Noticeably, the *M. cuneatum* accessions with intermediate features of calyx teeth (Figs. 5B, D, G, H) and the absence of sinuses between the calyx teeth (Figs. 5C, E) certainly support this polymorphism.

Based on taxonomic point of view and cluster analysis in *M.* sect. *Marrubium*, two groups were identified. The taxonomic status of the members was closely matched with Figure 3 using both sections (Fig. 7). In this cluster analysis most of the species such as *M. anisodon*, *M. vulgare* and *M. cuneatum* were definitely grouped but *M. vulgare* (with accession no. 38) was grouped with *M. crassidens* (with accession

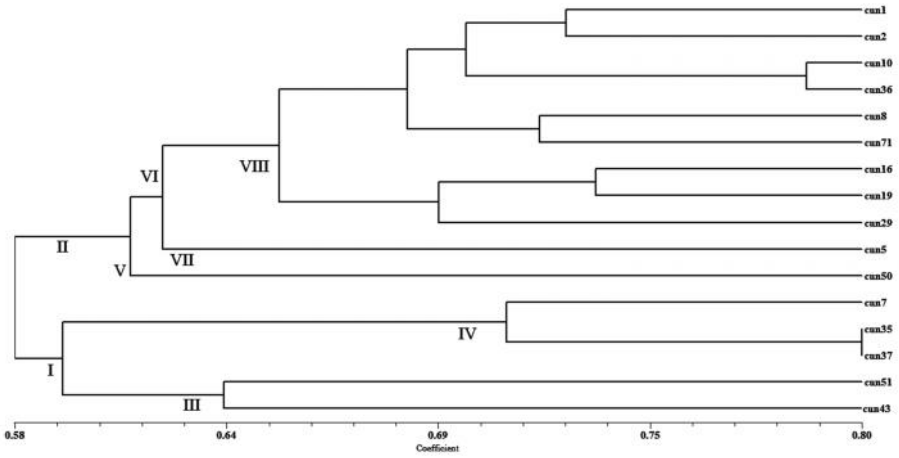

Figure 4. Dendrogram of sixteen *M. cuneatum* accessions using ISSR molecular markers

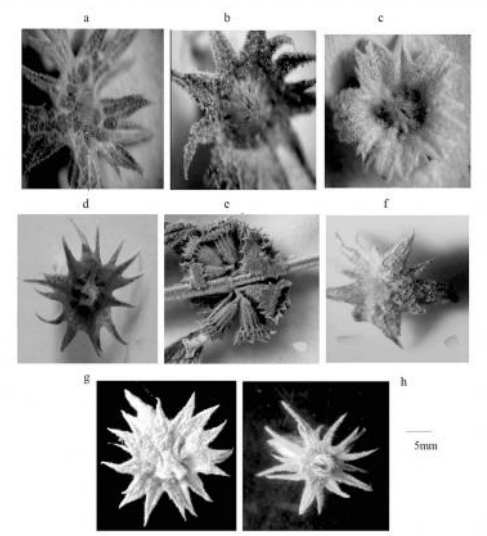

Figure 5. Forms of calyx teeth in *M. cuneatum* accessions X10

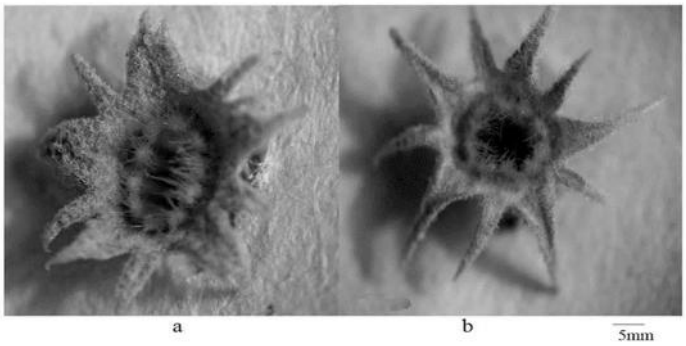

Figure 6. Forms of calyx teeth in *M. crassidens* var. *brevidens* accessions X10

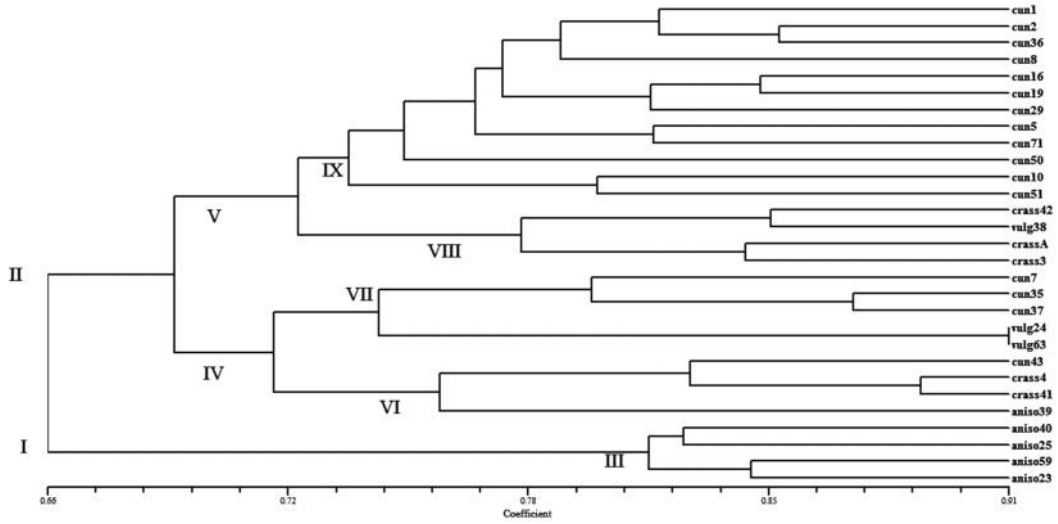

Figure 7. Dendrogram of *Marrubium* species belonging to *M.* sec. *Marrubium* using ISSR molecular markers. Cun: *M. cuneatum*, vulg: *M. vulgare*, crass: *M. crassidens*, aniso, *M. anisodon*, ballot: *Ballota aucheri*

no. 42) and *M. cuneatum* (with accession no. 43) was separately clustered with *M. crassidens* (with accession nos. 4 and 41).

Discussion

Based on the molecular cluster analysis, we found an exact position for the both sections; *M.* sect. *Marrubium* and *M.* sect. *Microdonta*. The genetic diversity results in Zagros region showed high variations in *M.* sect. *Marrubium*, *M.* sect. *Microdonta* and *B. aucheri* using cluster analysis. Indeed, *ISSR* molecular marker clearly provided variations in members of both sections (Figs. 3 and 7). It is attributed to *M. cuneatum* with 11 groups, *M. anisodon*, *M. crassidens* and *M. astracanicum* with three groups and *M. vulgare* with two groups (Figs. 3 and 7). *Kharazian* and *Hashemi* reported high morphological and flavonoid profile variations in the both sections. These variations were observed in *M. cuneatum*, *M. vulgare* and *M. anisodon* [21]. As mentioned above, these molecular variations were in agreement with previous results [21].

Marrubium cuneatum accessions exhibited ten different groups (Fig. 4) which are based on previous results of flavonoid and morphological studies from *Marrubium* species [21]. It is noted that the morphological variations in *M. cuneatum* accessions (Fig. 5) might be due to the polyploidy and hybridization features. It appears that some *Marrubium* species influence this feature. It is recognized by morphological character such as shape of calyx teeth. *Kharazian* and *Hashemi* pointed out these variations are especially focused on the number of flowers in each verticillaster (16-24), bract size (15-25 × 5-20 mm), length of calyx teeth (0.5-4 mm), number of calyx teeth

(12-30), the absence of sinuses between calyx teeth and type of coalition in the base of calyx teeth, corolla length (5.2-6.5 mm); indumentum of stem, leaf, inflorescence axis, bract, bracteole, calyx and corolla [21]. *Ahvazi* et al reported the variations of trichome length of calyx in *M. cuneatum* [3]. Noticeably, *M. cuneatum* with accession nos. 7, 35 and 37 with different morphological characters in terms of calyx teeth were grouped separately (Fig. 3). It is revealed by the absence of sinuses between the calyx teeth (shape of teeth) and the type of coalition at the base of them (Fig. 5e). Accession no. 29 was also placed in a different group which is characterized by the shape of calyx teeth (including two or more simple teeth) (Fig. 3). Molecular analyses evidently support the previous results [21]. Moreover, *M. cuneatum* was definitely separate from the other species. Molecular analysis of this study also confirmed these variations. High degree of polymorphism was detected including the genetic parameters such as Gst=0.86, I=0.55, Ne= 1.6, PP=89.8% and He= 0.37 (Tables 4 and 6).

It is noted that the applied technique in this study powerfully discriminated against *M. cuneatum* and *M. vulgare*. *Akgul* et al. reported that some of the pollen features, seed shape and seed ornamentation from *M. cuneatum* and *M. vulgare* were similar [4] and included closed groups involving high hybridization between them [21, 40]. However, they were different in regard to exine ornamentation, shape of polar/equatorial axes and seed dimensions [4]. Therefore it displayed two distinct clades which are strongly in accordance with our molecular results.

Some of the *M. crassidens* var. *brevidens* accessions showed broad calyx teeth in the base which strongly differ from the other accessions, i.e., the middle teeth of calyx. All of the calyx teeth were simple and slender but the only one had a bi-dentate lobe of calyx and wide base (Fig. 6). This difference was found in our clustering analysis with molecular data. *Marrubium crassidens* accessions were also distinctly grouped but the accession no. 42 was clustered with the *M. vulgare* accession (with accession no. 38) (Fig. 3). It can be stated with confidence that *M. crassidens* var. *brevidens* displays variability in its accessions. *Ahvazi* et al. observed mono-cellular simple hairs in upper surface of leaf in these species [3]. In addition, *M. crassidens* clustered near to *M. cuneatum* with accession no. 43 (Fig. 7) which is also substantiated using morphological reports [21, 40]. Controversially, flavonoid reports do not confirm this finding [20]. *M. crassidens* var. *brevidens* exhibits a substantial deviation in the formation of the calyx teeth [21, 40]. *Marrubium crassidens* related with *M. cuneatum* and distributed closely with *M. cuneatum* [40]. It is reasonable to assume that it would be an intermediate or hybrid species which is detected using *ISSR* molecular markers (Fig. 7). The molecular variations of this species were strongly detected by morphological and flavonoid data [21], but do not support morphological evidences of some previous results [40].

In another case, *Seybold*, *Jamzad*, *Ahvazi* et al. and *Kharazian* and *Hashemi* also reported high morphological variation and polymorphism for *M. astracanicum* which is supported by our molecular analysis results [3, 17, 21, 40]. As a result, this species is comprised of different groups (Fig. 3). *Akgul* et al. reported that the pollen features of *M. astracanicum* such as polar axis, polar/equatorial axes and shape, exine thickness, pollen meridional and ornamentation, and seed shape were different especially in comparison with *M. cuneatum* and *M. vulgare* [4] which is not based on our molecular data. Exceptionally, *M. cuneatum* with accession no. 43 was clustered with *M. astracanicum* but both of them definitely differed (Fig. 3). *Ahvazi* et al. also reported similar stellate trichomes in both species [3].

Marrubium anisodon and *M. cuneatum* detected different groups (Fig. 3). *Abu-Asab* and *Cantino* reported that *M. anisodon* and *M. cuneatum* were different in colpi, polar and equatorial axes, polar/equatorial shape, exine thickness, and seed sizes but were similar in pollen sculpturing of upper and lower, exine and seed ornamentation [1]. *Akgul* et al. also detected different pollen meridional in *M. cuneatum* [4]. Morphological features, micro-morphology of trichomes and flavonoid profiles from previous results powerfully confirm our molecular results [3, 20]. *Marrubium anisodon* accessions included distinct groups. *Marrubium anisodon* with accession no. 39 was also clustered as a separated group from the other *M. anisodon* accessions (Fig. 3). In terms of morphology and flavonoid profiles, *Kharazian* and *Hashemi* reported the differences of *M. anisodon* compared with the other *Marrubium* species [21]. Controversially, phylogenetic analysis showed close relations between *M. anisodon* and *M. vulgare* [4] which is comprised of two distinct clades. Our molecular results do not confirm previous researches [4]. It can be determined that *ISSR* markers strongly differed among *Marrubium* species.

Despite *Marrubium* presenting a complex group [4], *ISSR* molecular markers highlight the discriminating capacity to identify the taxonomic status.

In spite of high similarity between *B. aucheri* and *Marrubium* species, they form separate groups in cluster analysis which is confirmed by previous reports

[21].

Noticeably, molecular analysis of *M.* sect. *Marrubium* and *M.* sect. *Microdonta* illustrated a high degree of diversity and polymorphism which it presents through high genetic variations for all of the primers used such as Gst=0.98, He= 0.34, PP=100%, Ne= 1.53 and I=0.51 (Tables 3 and 6). Similarly, these polymorphisms were also separately detected in section *Marrubium* for example Gst=0.99, I=0.53, Ne= 1.57, He=0.35 and PP=99.1% (Tables 5 and 6). Based on previous results, the high value of genetic diversity, percentage of polymorphism, the Shannon information index and expected heterozygosity were found in different sections of the genus *Stachys* in Iran and Russia (Lamiaceae, Lamioideae) using *ISSR* molecular markers [20, 24] which supports our results.

Based on the molecular data, in the both sections PIC ranges from 0.17-0.45 and PP varies from 89.8-100%. Moreover, Gst ranges from 0.86-0.99 and Fis ranges from 0.01-0.14. *Kochieva* et al. and *Kharazian* et al. also detected a high percentage of polymorphism (315 polymorphic fragments; PP=71.18-100%) in different sections of *Stachys* [20, 24]. Moreover, *Song* et al., *Erbano* et al. and *Yousefi Azarkhanian* et al. reported high percentage of polymorphism and high genetic diversity in *Salvia* species [15, 42, 47]. Our molecular results were in accordance with previous studies in Lamiaceae members utilizing *RAPD* and *ISSR* markers [37, 42, 48].

Analysis of molecular variance (*AMOVA*) also showed high genetic variations among populations and low variations among species. *Wu* et al. reported high gene flow (Nm) among populations from *Rhododendron* L. species [46]. The high genetic diversity and low genetic differentiation in species can be explained by the life history traits, pollen dispersal and high gene flow. Pollen or seed dispersal over large distances cause high gene flow among populations. Wind dispersal over large distances can also influence it [46]. Gene flow among *Marrubium* populations was also found to be high (Nm=2.3) which coincides with *Wu* et al. [46]. The low genetic variation of species in small populations is less than in larger ones and this correlates to genetic drift and inbreeding systems. However, the small size of populations displays high genetic diversity. This is due to vegetative forms and habitat specifications. Moreover, outcrossing systems display higher genetic diversity than self-systems [46]. It is known that the genetic differentiation and population structure of some species do not correlate with geographical detachment [11]. Based on our findings, there is a low correlation between geographical and genetic distance which is based on *Chen* et al. [11].

Obviously, a strong disparity among populations was due to high Fst or Gst values and high genetic diversity. In our results, high levels of genetic diversity (Gst=0.86-0.99, Shannon information index=0.51-0.55, Ne= 1.53-1.6 and He=0.34-0.37) restricted the genetic differentiation which was detected by the *ISSR* technique and accomplished by the above mentioned results [37, 50]. The habitat fragmentation causes high differentiation among populations and leads to a strong genetic differentiation among them [49]. Moreover, there is increased genetic differentiation of species affected by repressed gene flow [44]. The genetic structure of regular populations is strongly affected by both fundamental factors [46]. On the whole, genetic differentiation would be controlled by vicariant factors [26]. Evidently, the species with a wider distribution tends to have higher genetic diversity [35]. However, further studies are needed to illustrate the breeding system. Thus, the levels of polymorphism at the *ISSR* loci from 35 *Marrubium* accessions are likely to give an accurate estimate of *Gst*.

In the both studied sections and *B. aucherii*, low levels of inbreeding coefficient (Fis) were found suggesting that the breeding system of *Marrubium* taxa encompasses high population and species diversity. Other molecular evidence such as marker index (MI) value ranges from 0.75-4.7, and the Shannon information index (I) ranges from 0.51-0.55 which supports the above results.

Conclusion

Based on these assumptions, the gene pool diversity can be ascribed to the rich genetic resources in the Zagros region which are significant in plant breeding. Estimating the high genetic diversity requires the recognition of regions with polymorphism and justifies organization of this germplasm [37]. To better understand the patterns of genetic diversity and structure on the quantitative genetic differentiation, breeding system and habitat fragmentation will be needed [35]. Because of the well-documented taxonomical status of *Marubium* species achieved through *ISSR* molecular markers, these are the tools of choice to identify the genetic diversity in *Marrubium*. It is recognized that molecular investigations discriminated *Marrubium* species in term of taxonomical point of view. The shape of calyx teeth in *M. cuneatum* and *M. crassidens* revealed high variations which is consistent with molecular results. It is observed that the other species of *Marrubium* do not display this variation. In addition, *M. cuneatum* and *M. crassidens* detected inter-specific hybridization.

Marrubium vulgare was closely related to *M. crassidens* but *M. anisodon* was definitely different from other *Marrubium* species. It is probably that *M. astracanicum* and *M. cuneatum* display different ecotypes in Zagros region, Iran.

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