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Phenotypic and Genotypic Assessment of Some Iranian *Ziziphora clinopodioides* Lam. Ecotypes

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ABSTRACT

***Ziziphora clinopodioides* Lam., a medicinal plant of Lamiaceae family, is a widespread species all over Iran. In this investigation, the genetic diversity of 10 ecotypes of *Ziziphora clinopodioides* was evaluated using morphological, phytochemical, and molecular markers. ISSR and iPBS markers were applied for the molecular analysis. The average of polymorphic bands per primer in the iPBS and ISSR markers were 4.4 and 6.68, with the average polymorphism of 79.49 and 92.03%, respectively. The ecotypes of Evard and Sorkhgarive had the highest values of the important breeding traits, including the shortest internode, the highest ratio of leaf to vegetative body weight, the highest essential oil yield, and the total phenol content. The results showed that ecotypes had a wide variation in terms of all studied markers. Principal Coordinate Analysis (PCoA) confirmed the results of molecular clustering of ecotypes, but phytochemical and morphological data did not have alignment with that. Also, there was no correspondence among the geographical locations of habitats and the phytochemical, morphological, and molecular markers data.**

Keywords: iPBS, ISSR, Genetic diversity, Medicinal plants, Morphological traits.

INTRODUCTION

Ziziphora clinopodioides Lam. is a medicinal plant belongs to the Lamiaceae family. The species is widely distributed in Iran and shows a high level of morphological diversity such that nine subspecies have been reported that widely grow in different climates and are highly affected by ecological conditions (Jamzad, 2012). Also, it has many ecotypes that are formed by multiple trait adaptations to many environmental variables (Lowry, 2012). This plant, named "Kakuti kouhi" in Iran, is used in Iranian traditional medicine for various therapeutic and culinary purposes (Ahvazi *et al*., 2012; Sheidai *et al*., 2019).

The plant phenotype is a result of genetic and environmental characteristics and their interactions (Khadivi-Khub *et al*., 2015). In some studies on *Zizphora* species, morphological markers were useful for the diagnosis of subspecies within the species. Keshavarzi *et al*. (2008) showed that micro-characters of epidermis were helpful in the taxonomy of *Z. clinopodioides* subspecies.

Based on morphological data in *Melissa officinalis* L., there was no correlation between the diversity of morphological features and the geographic region (Pouyanfar *et al*., 2018). Conversely, a relationship has been observed between morphological traits and ecological situations in *Ziziphora clinopodioides* (Asgharipour *et al*., 2016).

Under different ecological conditions, the quantity and the quality of the plant secondary metabolites might be adapted to these conditions. Many studies have shown that the composition and content of various populations of a species in different

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geographic regions are different. It has been reported that the chemical profile of the genus *Ziziphora* is influenced by various climate conditions, such that the presence of the chemotypes has been observed (Alp *et al*., 2016; Ebrahimi *et al*., 2012; Aghajani *et al*., 2008; Khadivi-Khub *et al*., 2014; Sonboli *et al*., 2010).

Molecular markers are useful tools for evaluating the genetic diversity and evolutionary relationships in plants. ISSR and iPBS as highly polymorphic marker techniques are used to investigate the genetic diversity of natural populations, the identification of cultivars, the description of germplasm, and phylogenetic studies (Dalir and Safarnejad, 2017; Hadian *et al*., 2017; Tabaripoor *et al*., 2016; Kalendar *et al*., 2010). ISSR marker has been efficiently used for the analysis of population structure and genetic diversity of *Z. tenuior* (Tabaripoor *et al*., 2016; Dakah *et al*., 2015). Also, this marker has been used to study genetic diversity and population structure of different species such as *Satureja bakhtiarica* (Saidi *et al*., 2013), *Lallemantia iberica* (Koohdar *et al*., 2015), *Mentha cervina* (Rodrigues *et al*., 2013) and *Salvia hispanica* (Palma-rojas *et al*., 2017).

The use of iPBS marker in investigating species such as *Leonurus cardiaca* L. (Borna *et al*., 2017), *Coriandrum sativum* L. (Alp and Gebologlu, 2017), *Camellia sinensis* L. (Phong *et al*., 2016), *Pisum sativum* (Baloch *et al*., 2015) has been successful.

Both of the markers, ISSR and iPBS, do not require prior information about the genome sequences. Since we did not have any previous information on the genome sequences of this plant, we used these markers in this research. Preliminary studies have been done on the phytochemical properties of this plant, but no molecular study has been done. Despite the wide variety and overlapping of the subspecies, a comprehensive study is required using molecular methods. Therefore, for the first time, we tried to assess morphological, phytochemical, and molecular diversity of some populations in *Z. clinopodioides*. Geographically distant ecotypes were selected to have enough isolation and better diversity. The objective of our study was the comparison of 10 ecotypes originating from different Iranian regions to provide useful information for the future conservation of the domesticated resources and breeding programs of *Z. clinopodioides.*

MATERIALS AND METHODS

Plant Material

A total of ten wild ecotypes of *Z. clinopodioides* were collected across seven provinces of Iran. Sampling locations are shown in Table 1. The aerial parts of plants (stem, leaves, and flowers) were collected during the flowering stage in July and August of 2017. Samples were wrapped stored in ice to avoid oxidation. Then, they were stored in a freezer at -80°C. The botanical identity of the species was authenticated using Flora of Iran (Jamzad, 2012), and the voucher specimens were deposited in the Herbarium of the Horticultural Department, University of Tehran (Table 1).

Morphological Study

Each ecotype was assayed for morphological markers by measuring fifteen traits in 10 individual plants. The morphological traits studied included leaf, stem, flower, and inflorescence, which are mentioned in Table 4.

Phytochemical Study

Essential Oil Extraction

Aerial parts of the plants were dried for one week at room temperature (20-25°C) in the shade.

The plant material (100 g) was subjected to hydro distillation for three hours, using a

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Table 1. Locations of the studied ecotypes of *Ziziphora clinopodioides* in Iran.

No	Province	Ecotype	Vouchers no	Altitude (m)	Longitude (E)	Latitude (N)
1	Chaharmahal-o-Bakhtiyari	Boroujen	006469	2,300	31°56′3.12”	51°3′28.08”
2	Qazvin	Qazvin	006470	1,900	36°25′08.3”	50°05′54.2”
3	Markazi	Shazand	006472	1,950	33°55′16.3”	49°23′54.1”
4	Mazandaran	Sorkhgarive	006474	1,900	36°33′32.2”	54°03′09.8”
5	Mazandaran	Evard	006475	1,300	36°37′28.6”	53°47′03.7”
6	Khorasan Shomali	Gardane tivan	006471	1,100	37°35′16.30”	58°37′58.81”
7	Khorasan Shomali	Bajgiran	006476	1,638	37°37′16.30”	58°25′29.84”
8	Ilam	Ilam	006468	1,840	33°08′54.96”	47°8′39.89”
9	Tehran	Polour	006473	2,200	35°50′50.99”	52°03′9.28”
10	Alborz	Gajre	006467	2,510	36°3′44.27”	51°23′24.66”

Table 2. List of ISSR primers used in the molecular study of *Ziziphora clinopodioides* ecotypes.

No	Primer	Sequence (5′-3′)	TM (°C)
1	ISCS7	*TCTCTCTCTCTCTCTCTCC*	60.1
2	ISCS10	*ACACACACACACACACC*	52
3	ISCS12	*TTGTTGTTGTTGTTGTTGC*	52.2
4	ISCS17	*DBDBCACCACCACCACCAC*	64.9
5	ISCS30	*ACACACACACACACACYT*	54.5
6	ISCS43	*GAAGAAGAAGAAGAAGAA*	54.3
7	ISCS47	*CACACACACACACACARG*	52
8	ISCS51	*CACACACACACACACART*	49.3
9	ISCS58	*GAGAGAGAGAGAGAGAYC*	58.2
10	ISCS64	*GAGAGAGAGAGAGAGAC*	49.3
11	ISCS65	*GAGAGAGAGAGAGAGAA*	52.5
12	ISCS69	*CACACACACACACACAA*	52.7
13	ISCS70	*CACACACACACACACAG*	58.2
14	ISCS87	*AGAGAGAGAGAGAGAGYA*	56.4
15	ISCS18	*DBDBCCACCACCACCACCA*	54.7
16	ISCS32	*ACACACACACACACACYG*	54.5
17	ISCS50	*CACACACACACACACARC*	59
18	ISCS73	*GTGTGTGTGTGTGTGTT*	49
19	ISCS77	*T ACACACACACACACACT*	49

Table 3. List of iPBS primers used in the molecular study of *Ziziphora clinopodioides* ecotypes.

No	Primer	Sequence (5′-3′)	Ta (°C)
1	2076	*GCTCCGATGCCA*	54
2	2389	*ACATCCTTCCCA*	40.4
3	2380	*CAACCTGATCCA*	40.4
4	2390	*GCAACAACCCCA*	45
5	2391	*ATCTGTCAGCCA*	34
6	2083	*CTTCTAGCGCCA*	39.5
7	2382	*TGTTGGCTTCCA*	41

Clevenger-type apparatus according to the method recommended by British Pharmacopoeia (1993). The collected oils were stored in dark vials at 4°C until the analysis.

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Methanol Extract

Plants were powdered (20 g) and macerated with methanol (1:10, w/v) at room temperature for 72 hours. The aliquots were occasionally shaken. Then, the extracts were filtered. This process was repeated until exhausted (Azwanida, 2015).

Total Phenolic Compounds Content

The content of total phenolic compounds was determined by Folin-Ciocalteu's protocol described by Slinkard and Singleton (1977). For this purpose, 20 µL of the extract solution was mixed with 1.58 mL of distilled water and 100 mL of Folin–Ciocalteu reagent and gently shook the cuvette. After three min, 300 mL of sodium carbonate solution (7% w/v) was added. The mixture was left for two min at room temperature with occasional shaking. Absorbance was measured at 760 nm wavelength by spectrophotometer. Total phenolic content was expressed as grams of gallic acid equivalents in one gram of dry weight.

Total Flavonoid Compounds Content

The total flavonoid content was measured according to the aluminum chloride colorimetric method (Quettier and Deleu, 2000). An aliquot of the methanolic extract (500 µL) was added to 500 µL of 2% aluminum chloride solution. The mixture was allowed to stand for 10 minutes at room temperature with occasional shaking. The absorbance of the samples was quantified at 415 nm by a spectrophotometer. A blank sample was the aliquot without aluminum chloride. Total flavonoid content was recorded as grams of Rutin equivalents in one gram of dry weight.

Antioxidant Activity

Antioxidant activity of a metabolite is characterized by its free radical scavenging activity. DPPH (2, 2-diphenyl-1-picrylhydrazyl) test was used to determine the free radical-scavenging activities of the extracts (Gholivand *et al*., 2014). Three mL of various concentrations of the extract were mixed with a 1 mL methanol solution of DPPH (0.5 mmol L^{-1}). The resulting solution was shaken and allowed to stand 60 minutes at room temperature. The absorbance of samples was recorded at 517 nm wavelength against that of the blank. The percentage of free radical-scavenging activity (I) was calculated by the following formula:

$I\% = [(Ab–AS)/Ab]\times100$

Where, the Absorbance of the control reaction is Ab, and the Absorbance of the test compound is As. The concentrations of the samples caused by 50% inhibition (IC50 value) were measured by linear regression analysis obtained from sample values. The positive control was Ascorbic Acid (AA) (Gholivand *et al*., 2014).

Molecular Study

DNA Extraction

Young leaves from 10 individual plants of each ecotype were taken and pooled in equal weight, then, 0.2 g of them were used for genomic DNA extraction. The DNA extraction protocol was based on a modified CTAB (cetyltrimethylammonium bromide) method (Doyle, 1991). The quality and quantity of DNA were checked using both nanodrop spectrophotometer and electrophoresis. The DNA was diluted to an operating concentration of 10 ng μL^{-1}.

ISSR-PCR Amplification

Twenty ISSR primers were screened and a total of nineteen that showed high polymorphism was selected for PCR analysis (Table 2). The PCR reaction was carried out in a total volume of 15 µL containing 7.5 µL of the Master Mix Red (Ampliqon) including (dNTPs, MgCl2, Taq

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DNA polymerase), 3 µL of distilled water, 2.5 µL of total DNA(10 ng μL^{-1}), and 2µL of each primer. PCR amplifications were set as initially for 5 minutes at 94°C followed by 35 cycles with 50 seconds (denaturation) at 94°C, annealing (at the temperature shown in Table 2) for 1 minute, extension at 72°C for 2.5 minutes, and finally an extension cycle of 5 minutes at 72°C. DNA amplification fragments were separated by electrophoresis on a 1.2% (w/v) agarose gel using a 10X TAE buffer at 80 V cm^{-1} for 150 minutes, then photographed using Gel Doc (BioDoc-1t TM System UPV).

iPBS-PCR Amplification

A total of seven prescreened and selected iPBS primers was used for PCR amplification (Table 3). A fifteen µL of PCR mixture contained 7.5 µL of the Master Mix Red (Ampliqon Co.) including (dNTPs, MgCl2, Taq DNA polymerase), 3 µL of distilled water, 2.5 µL of total DNA(10 ng μL^{-1}), and 2 µL of each primer. The PCR conditions were as follows: initial denaturation at 94°C for 30 seconds; 49 cycles at 94°C for 30 seconds, the 30 seconds at defined annealing temperature (31-69°C), and 72°C for 2 minutes; and a final extension at 72°C for 5 minutes. PCR products were electrophoresed in 1.5% agarose gel using a 10X TAE buffer at 80 V cm^{-1} for 180 minutes and then observed using Gel Doc (BioDoc-1t TM System UPV).

Statistical Analysis

Morphological and phytochemical data were subjected to Analysis Of Variance (ANOVA) with three replications, and clustering was run by the software of SPSS v.22 based on standardized data using square euclidean distance (Norusis, 2011). The means comparison was performed using the LSD test with a significance threshold of 0.01.

The amplified bands were scored as 1 for the presence and 0 for the absence of a band. The POPGENE 1.31 software was used for analyzing the data to estimate the diversity parameters. Principal Coordinate Analysis (PCoA) and Mantel test were conducted by the software of GenAlex Ver. 6.4. The dendrogram was constructed by distance matrix using the UPGMA method by NTSYS-pc 2.10 software. Polymorphic Information Content (PIC) was calculated according to Singh and Singh (2015):

PIC = $\sum 2p_i q_i$

Where, p and q were allele frequencies.

RESULTS

Phenotypic Assessment

Significant differences in all evaluated traits were found by the ANOVA. The results of the mean comparison of phenotypic traits are presented in Table 4. The ecotypes of Evard and Polour had the lowest (18.5 cm, 1.72 cm, and 14.79) and the highest (37.5 cm, 4.02 cm, and 86.5) values for stem length, internode length, and the number of flowers, respectively. The ecotype of Evard also showed the lowest values (0.64, 0.32, and 0.84 cm) for leaf length, leaf width, and inflorescence length, respectively. The highest (65%) and the lowest (38%) values of leaf weight to whole plant weight ratio were obtained in the ecotypes of Evard and Boroujen, respectively.

Grouping the ecotypes based on the all phenotypic attributes was done by cluster analysis (Figure 1). The ecotypes were divided into two main groups, as group A consisted of two sub-clusters. The sub-cluster A1 contained Bajgiran, Gardaneh tivan, and Shazand ecotypes. The second sub-cluster (A2) included Gajre, Polour, Ilam, Qazvin, and Boroujen ecotypes. The group B also comprised Evard and Sorkhgarive ecotypes. As noted in Table 5 results, these ecotypes had a negative deviation from the total mean and the lowest values of most of the studied traits.

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Table 4. Mean comparisons of the ecotypes of *Ziziphora clinopodioides* for morphological traits. [a]

Traits	Ecotype									
	Boroujen	Qazvin	Shazand	Sorkhgarive	Evard	Gardane tivan	Bajgiran	Ilam	Polour	Gajre
Leaf length (cm)	1.51^{c}	1.43^{c}	2.61^{a}	0.92^{d}	0.64^{d}	1.54^{c}	1.76^{bc}	1.64^{bc}	1.9^{b}	1.75^{bc}
Leaf width (cm)	0.34^{d}	0.52^{bc}	0.52^{bc}	0.53^{bc}	0.32^{d}	0.54^{bc}	0.65^{a}	0.50^{c}	0.58^{ab}	0.48^{c}
Internode length (cm)	3^{b}	2.9^{bc}	3.77^{a}	1.73^{d}	1.72^{d}	2.46^{c}	2.39^{c}	3.15^{b}	4.02^{a}	3.07^{b}
Stem length (cm)	25.63^{c}	23.03^{d}	25.92^{c}	19.06^{f}	18.5^{f}	21.13^{e}	18.75^{f}	27.15^{b}	37.50^{a}	28.07^{b}
Inflorescence length (cm)	1.30^{b}	1.33^{b}	1.68^{a}	1.36^{b}	0.84^{c}	1.29^{b}	1.32^{b}	1.32^{b}	1.66^{a}	1.76^{a}
Number of flower (no)	35.06^{e}	36.30^{e}	55^{c}	24.48^{f}	14.79^{g}	43.04^{d}	42.54^{d}	58.55^{c}	86.50^{a}	68.54^{b}
Inflorescence leaf length (cm)	0.63^{cde}	0.62^{cde}	1.61^{a}	0.54^{e}	0.36^{f}	0.98^{b}	1.08^{b}	0.60^{de}	0.80^{c}	0.77^{cd}
Inflorescence leaf width (cm)	0.15^{e}	0.25^{d}	0.46^{b}	0.34^{c}	0.18^{de}	0.48^{b}	0.56^{a}	0.35^{c}	0.35^{c}	0.34^{c}
Corolla length (cm)	0.56^{a}	0.35^{ef}	0.47^{bc}	0.38^{def}	0.33^{f}	0.44^{cd}	0.47^{bc}	0.53^{ab}	0.38^{def}	0.42^{cde}
Calyx length (cm)	0.42^{e}	0.48^{bcde}	0.51^{abcd}	0.57^{a}	0.45^{cde}	0.52^{abc}	0.55^{ab}	0.44^{de}	0.41^{e}	0.42^{e}
Number of lateral branches (no)	4.60^{bcd}	4.23^{cde}	2.16^{g}	9.46^{a}	9.45^{a}	5.04^{bc}	4.03^{de}	3.54^{ef}	5.50^{b}	3.02^{fg}
Lateral branches length (cm)	6.10^{cd}	12.72^{a}	6.60^{c}	6.71^{c}	9.12^{b}	5.52^{d}	5.80^{cd}	6.54^{c}	6.20^{cd}	5.46^{d}
Leaf length to leaf width ratio	4.48^{ab}	2.76^{cde}	4.97^{a}	1.73^{f}	1.97^{ef}	2.83^{cde}	2.72^{de}	3.31^{cd}	3.25^{cd}	3.64^{bc}
Inflorescence leaf length to Inflorescence leaf width ratio	4.28^{a}	2.45^{c}	3.46^{b}	1.58^{d}	1.94^{cd}	2.05^{cd}	1.93^{cd}	1.71^{cd}	2.28^{cd}	2.28^{cd}
Leaf weight to whole plant weight ratio (%)	38^{i}	42^{g}	55^{d}	57^{c}	65^{a}	51^{e}	47^{f}	59^{b}	50^{e}	40^{h}

[a] Different letters represent statistically different values for $p \leq 0.01$ (LSD test).

Table 5. The groups' averages and deviations from the total mean for morphological clustering in *Z. clinopodioides*.

Clusters	Traits															
	Leaf length		Leaf width		Internode length		Stem length		Inflorescence length		Number of flower		Inflorescence leaf length		Inflorescence leaf width	
	Mean	SD[a]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
A1	1.97	0.4	0.57	0.08	2.87	0.05	21.93	-2.54	1.43	0.05	46.86	3.38	1.22	0.43	0.5	0.16
A2	1.64	0.07	0.49	-0.01	3.22	0.4	28.27	3.8	1.47	0.09	50.99	7.51	0.68	-0.11	0.28	-0.06
B	0.78	-0.79	0.48	-0.07	1.72	-1.1	18.78	-5.69	1.1	-0.28	19.63	-23.85	0.45	-0.34	0.26	-0.08
Total	1.57		0.49		2.82		24.47		1.38		43.48		0.79		0.34	

Clusters	Traits															
	Corolla length		Calyx length		Number of lateral branches		Lateral branches length		Leaf length to leaf width ratio		Inflorescence leaf length to inflorescence leaf width ratio		Leaf weight to whole plant weight ratio (%)		Corolla length	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
A1	0.46	0.03	0.52	0.05	3.74	-1.36	5.97	-1.1	3.50	0.34	2.48	0.09	51	0.6	A1	0.46
A2	0.44	0.01	0.43	-0.04	4.17	-0.93	7.4	0.33	3.48	0.32	2.6	0.21	45.8	-4.6	A2	0.44
B	0.35	-0.08	0.51	0.04	9.45	4.35	7.91	0.84	1.85	-1.31	1.76	-0.63	61	10.6	B	0.35
Total	0.43		0.047		5.1		7.07		3.16		2.39		50.4		Total	

[a] Standard Deviation.

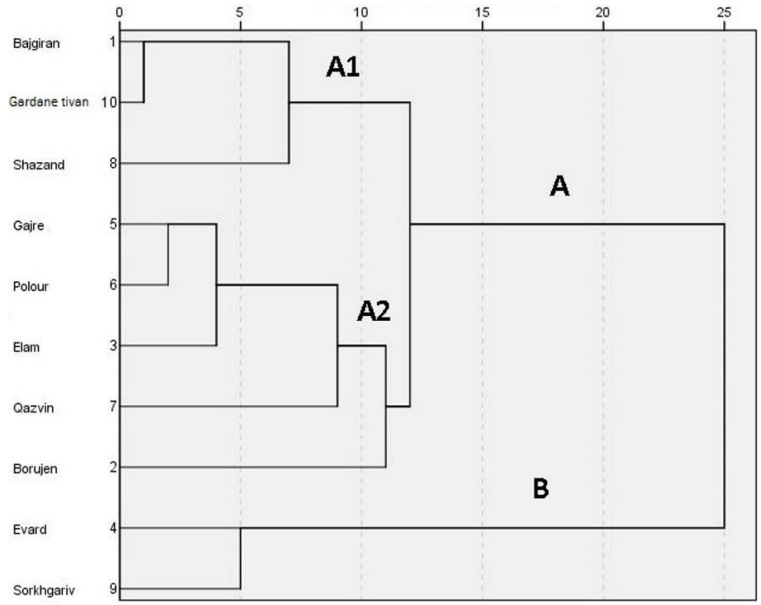

Figure 1. Clustering of the ecotypes of *Ziziphra clinopoides* based on the morphological traits by the complete linkage method.

Phytochemical Assay

The means of the ecotypes for the phytochemical attributes shown in Table 6 revealed that the ecotype of Evard had the highest values for total phenol content (82.43 mg GAE g^{-1}) and essential oil yield (2.1%). The lowest values of those belonged to the ecotypes of Polour and Boroujen (32.06 mg GAE g^{-1} and 0.47%). Evard and Gajreh revealed the lowest (45.99 mg Rutin g^{-1}) and the highest (78.93 mg Rutin g^{-1}) values of the total flavonoid content, respectively. The highest (80.38%) and the lowest (65%) values of the antioxidant activity were observed in the ecotypes of Gardaneh tivan and Qazvin, respectively.

Different letters represent statistically different values for $P \leq 0.01$ (LSD test).

Phytochemical clustering was run using the complete linkage method, which categorized the ecotypes into two main groups (A and B) (Figure 2). Group A was divided into two sub-clusters. Sub-cluster A1 contained Shazand, Polour, Boroujen, and Qazvin ecotypes, while sub-cluster A2 consisted of the ecotypes of Ilam, Sorkhgarive, Evard, and Bajgiran. The remaining ecotypes, Gajreh, and Gardaneh tivan, were in a distinct cluster, group B.

Molecular Analysis

iPBS

The selected seven iPBS primers generated a total of 39 scorable bands, in which 31 (79.49%) bands were polymorphic (Table 7). The average of polymorphic bands per primer was 4.4 and ranged from 1 (primer of 2391) to 7 (primer of 2380). The average Polymorphic Information Content (PIC) value was 0.41 (Table 7), with the primers of 2389 and 2391 having the highest (0.49) and the lowest (0.39) PIC values, respectively. Gene diversity index (h) of the primers varied from 0.03 (primer of 2391) to 0.34 (primer of 2389) with a mean value of 0.24. The average of Shannon's information index was 0.38, and the mean values ranged from 0.06 (primer of 2391) to 0.49 (primers of 2382, and 2389). The mean value for the effective number of alleles was 1.39.

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Table 6. Mean comparison of phytochemical traits in the ecotypes of *Z. clinopodioides.* [a]

Ecotype	Traits				
	Total phenol content (mg GAE g^{-1})	Total flavonoid content (mg Rutin g^{-1})	Antioxidant activity (%)	Antioxidant activity (IC50; mg mL^{-1})	Essential oil yield (%)
Borouje	56.7^{cd}	52^{cd}	69.62^{bc}	4.91^{bc}	0.47^{f}
Qazvin	52.66^{d}	59.37^{bc}	65^{c}	4.5^{c}	1.03^{e}
Shazand	34.23^{e}	49.87^{cd}	72.19^{bc}	5.23^{bc}	1.48^{cd}
Sorkhgarive	72.35^{ab}	54.56^{cd}	71^{bc}	5.1^{bc}	1.55^{c}
Evard	82.43^{a}	45.99^{d}	74.76^{ab}	5.45^{ab}	2.1^{a}
Gardane tivan	49.56^{d}	67.38^{b}	80.38^{a}	6^{a}	1.4^{d}
Bajgiran	64.6^{bc}	59.76^{bc}	73.76^{ab}	5.34^{ab}	1.1^{e}
Ilam	54.21^{d}	57.75^{bc}	71.43^{bc}	5.15^{bc}	1.74^{b}
Polour	32.06^{e}	57.5^{bc}	72.86^{b}	5.62^{b}	1.14^{e}
Gajre	57.59^{cd}	78.93^{a}	74.19^{ab}	5.41^{ab}	0.53^{f}

[a] Different letters represent statistically different values for P≤ 0.01 (LSD test).

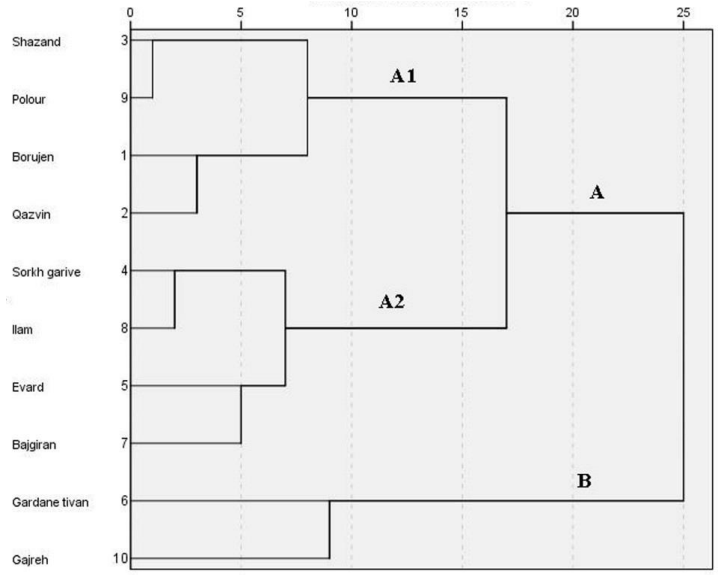

Figure 2. Cluster analysis of the *Ziziphora clinopodioides* ecotypes from Iran using phytochemical traits by the complete linkage method.

Table 7. The features of iPBS primers used for the analysis of genetic diversity in *Z. clinopodioides.*[a]

Primer	Total bands	Polymorphic bands	Polymorphism (%)	PIC	Ne	Na	h	I
2076	4	3	75	0.495	1.22	1.75	0.17	0.28
2389	8	7	87	0.463188	1.43	1.87	0.27	0.42
2380	6	6	100	0.48	1.53	2	0.32	0.49
2390	6	6	100	0.433022	1.42	2	0.29	0.46
2391	6	5	83	0.497822	1.59	1.83	0.34	0.49
2083	4	3	75	0.48	1.34	1.75	0.21	0.33
2382	5	1	20	0.0392	1.04	1.2	0.03	0.06
Mean	5.5	4.42	79.49	0.41	1.39	1.79	0.24	0.38

[a] PIC: Polymorphic Information Content; Na: Number of different alleles; Ne: Number of effective alleles; I: Shannon's information index, h: Gene diversity.

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Principal Coordinates Analysis (PCoA) showed the variability among the ecotypes in a two-dimensional model. (Figure 3). The first three components accounted for 59.85% of total variation observed in the ecotypes (First axis

The UPGMA dendrogram based on Nei's genetic distance obtained from iPBS markers data (Figure 4). The dendrogram grouped ecotypes into four main clusters. The first cluster (A) included Boroujen, Qazvin, and Shazand ecotypes. Cluster B comprised the ecotypes of Sorkhgarive, Ilam, Polour, and Gajre. Cluster C included only Gardane tivan ecotype. Evard and Bajgiran were clustered in a distinct group (D).

ISSR

ISSR primers produced 138 bands. Out of these, 127 bands (92.03%) were polymorphic. The number of polymorphic bands per primer varied from 1 (ISCS69) to 12 (ISCS64), with an average of 6.68 bands per primer (Table 8). The highest and the lowest PIC values for the amplification products were 0.49 (ISCS10, 32, 47, 58, 65, and 73) and 0.27 (ISCS69), respectively, with an average of 0.44. Gene diversity (h) per primer ranged from 0.16 (ISCS69) to 0.43 (ISCS73), with an average of 0.29. The mean of Shannon's information index was 0.45, ranging from 0.23 (ISCS69) to 0.61 (ISCS73). The mean value for the effective number of alleles was 1.47. The PCoA using ISSR data revealed that the first three components accounted for 48.07% of the total variation observed in the ecotypes (First axis= 19.66%, Second= 14.97% and Third= 13.43%) (Figure 5).

The results of PcoA also supported the results of the UPGMA dendrogram. Based on the

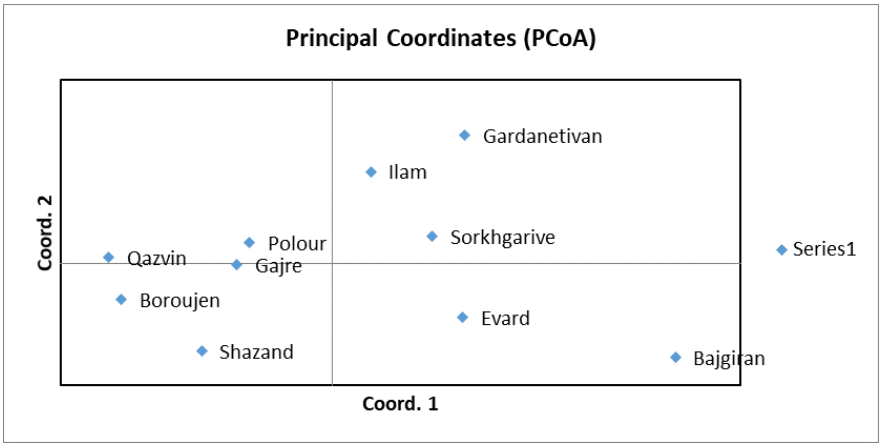

Figure 3. Principal coordinate analysis of the ecotypes of *Ziziphora clinopodioides* based on iPBS markers data.

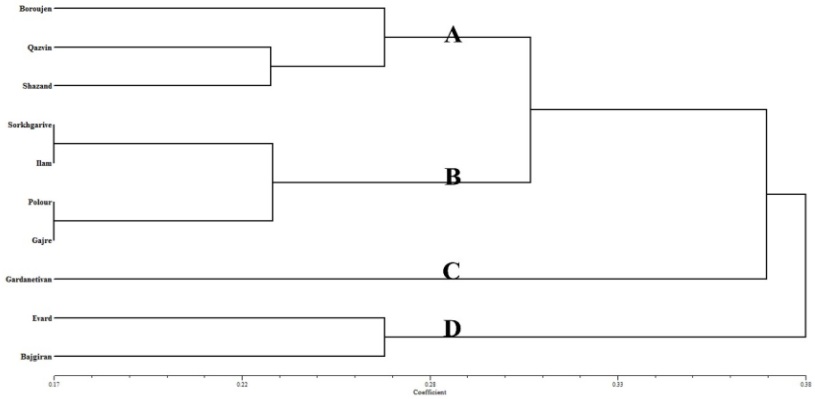

Figure 4. Dendrogram of the ecotypes of *Ziziphora clinopodioides* using iPBS molecular data.

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results, Boroujen, Qazvin, Shazand, Gajre, and Sorkhgarive ecotypes were classified in the close groups. Ilam ecotype was more separated from the other ecotypes.

A dendrogram was produced using Nei's genetic distance based on ISSR data (Figure 6). All the ecotypes were classified into four main groups. Group A included the ecotypes of Boroujen, Qazvin, Shazand, Sorkhgarive, Polour, and Gajre. Group B comprised of Gardane tivan and Bajgiran. Group C was single and consisted of ecotype Evard. The Ilam ecotype was in the last group.

Table 8. The efficiency and polymorphism of ISSR primers used for the analysis of genetic diversity in *Ziziphora clinopodioides*.[a]

Primer	Total bands	Polymorphic bands	Polymorphism (%)	PIC	Ne	Na	h	I
ISCS7	11	11	100	0.36	1.42	2	0.27	0.44
ISCS10	9	8	88.88	0.49	1.66	1.88	0.37	0.54
ISCS12	9	9	100	0.48	1.51	2	0.31	0.48
ISCS17	6	5	83.33	0.48	1.35	1.83	0.23	0.37
ISCS30	6	5	83	0.44	1.39	1.83	0.29	0.4
ISCS43	9	9	100	0.47	1.49	2	0.31	0.48
ISCS47	5	4	80.00	0.49	1.27	1.8	0.19	0.31
ISCS51	11	11	100	0.46	1.47	2	0.3	0.47
ISCS58	6	6	100	0.49	1.59	2	0.34	0.52
ISCS64	7	7	100	0.42	1.35	2	0.24	0.4
ISCS65	6	6	100	0.45	1.5	2	0.31	0.48
ISCS69	5	4	80	0.49	1.55	1.8	0.31	0.45
ISCS70	12	12	100	0.44	1.5	2	0.31	0.48
ISCS87	9	8	89	0.49	1.44	1.88	0.27	0.42
ISCS18	3	1	33.33	0.27	1.33	1.33	0.16	0.23
ISCS32	5	3	60	0.42	1.38	1.6	0.22	0.33
ISCS50	7	7	100	0.49	1.78	2	0.43	0.61
ISCS73	6	5	83.33	0.42	1.38	1.83	0.24	0.38
ISCS77	6	6	100	0.46	1.46	2	0.29	0.46
Mean	7.2	6.6	92.03	0.44	1.47	1.92	0.29	0.45

[a] PIC: Polymorphic Information Content; Na: Number of different alleles; Ne: Number of effective alleles; I: Shannon's information index, h: Gene diversity.

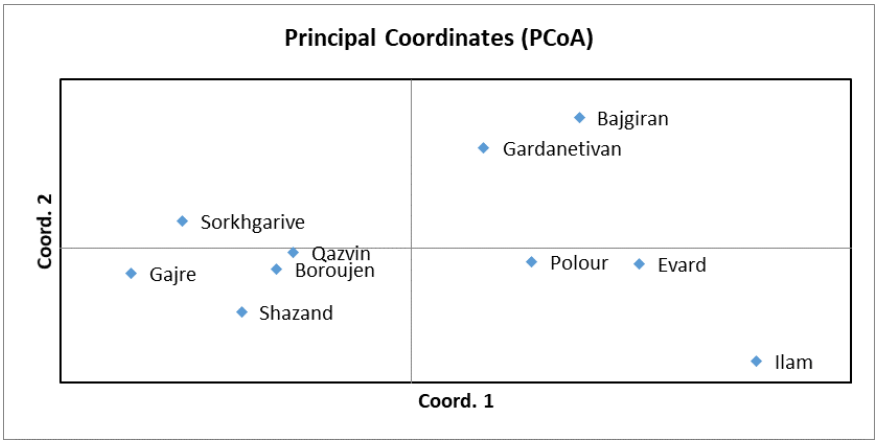

Figure 5. Principal coordinate analysis of the ecotypes of *Ziziphora clinopodioides* using ISSR data.

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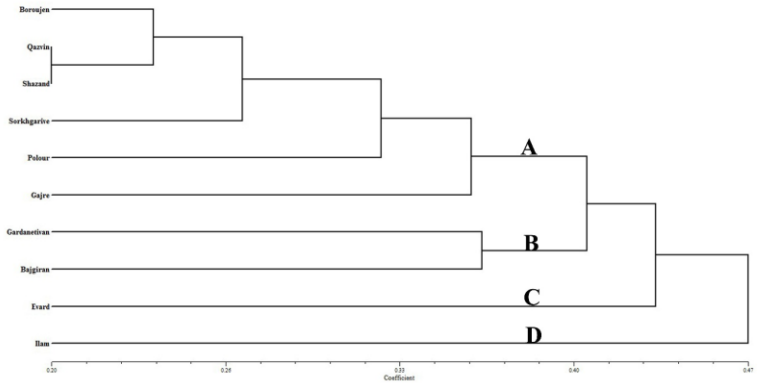

Figure 6. Dendrogram of the ecotypes of *Ziziphora clinopodioides* using ISSR data.

DISCUSSION

In the present study, morphological, phytochemical, and molecular markers were used to assess relationships among the ecotypes of *Ziziphora clinopodioides* from Iran. This is the first report on the comprehensive evaluation of the genetic relationships in the plant. The significant differences in the morphological and phytochemical data may be caused by various habitat conditions of the studied ecotypes. Several studies have reported the same results in other Lamiaceae plants, including *Z. clinopodioides* (Asgharipour *et al*., 2016), *Ziziphora tenuior* L. (Tabaripoor *et al*., 2016), *Stachys lavandulifolia* Vahl. (Arabsalehi *et al*., 2018), and *Thymus* L. (Dalir and Safarnejad, 2017). High variation in plants is important for doing breeding projects (Dalir and Safarnejad, 2017). According to the results, the ecotypes of Evard and Sorkhgarive had the best breeding traits, including the shortest internode, the highest ratio of leaf to vegetative body weight, the highest essential oil yield, and the total phenol content. Polour ecotype also had the highest number of flower and stem length, which are important in seeds production and facilitating mechanical harvest, respectively.

Classification of the ecotypes using phytochemical traits did not follow a specific pattern correspondence in geographical distribution. This is following earlier studies, e.g. *Artemisia herba-alba* (Younsi *et al*., 2018) and *Mentha cervina* (Rodrigues *et al*., 2013).

The Mantel test revealed that there was no correlation between morphological classification and climatic differences (r= 0.002; P= 0.410; data not shown), which indicated morphological traits might be influenced by different kinds of factors. There was the same result in *Z. tenuior* L. (Hatari *et al*., 2013), which showed no relation between morphological and geographic sites. Some other studies demonstrated a positive association between morphological and geographic locations in *Z. clinopodioides* (Asgharipour *et al*., 2016), *Perovskia abrotanoides* Karel. and *P. atriplicifolia* (Hashemifar and Rahimmalek, 2018).

Both of the studied molecular markers showed a high polymorphic percentage among the ecotypes. The generated polymorphism is an indicator for measuring the diversity of DNA sequences in the whole genome. The lower percentage of polymorphism for iPBS (79.49%) than ISSR marker (92.03%) could not be a valid reason for the ineffectiveness of this marker. The number and combination of primers used can be very effective on the results, but

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according to the average number of bands for each primer, it can be concluded that the ISSR marker had a better performance: for the ISSR marker, the average band for each primer was 7.2 bands, while for iPBS it was 5.5.

ISSR polymorphism in the present study was higher than those reported for some other species in Lamiaceae, such as *Ziziphora tenuior* L. (59%) (Tabaripoor *et al*., 2016), *Thymus* L. (83.22 %) (Dalir and Safarnejad, 2017), *Perovskia abrotanoides* KAR. (80.70%) (Pourhosseini *et al*., 2018), *Thymus caramanicus* (82.68 %) (Hadian *et al*., 2014) and *Thymus daenensis* (88.9%) (Rahimmalek *et al*., 2009). Also, iPBS polymorphism in previous studies on other plants like *Leonurus cardiaca* L. (52.40%) (Borna *et al*., 2017), *Pisum sativum* (76.4%) (Baloch *et al*., 2015), and *Abelmoschus esculentus* (40.2%) (Yildiz *et al*., 2015) was lower than the present study.

Gene diversity (h) for both markers was 0.28, and Shannon's information index was 0.43, which showed that there was a high variation between the ecotypes concerning both markers. Also, the ISSR marker had the highest values for gene diversity (h) and Shannon's information index. Moreover, the mean value of the PIC in this study was 0.42, which showed that the primers could produce acceptable polymorphism, which is useful for genetic variation of ecotypes studied in this research. A similar study using ISSR and iPBS markers reported the average PIC values higher than 0.5 in *Tetradium ruticarpum*, which show high loci polymorphism (XU *et al*., 2018).

There was no correlation between molecular clustering and geographical distribution of the ecotypes. These results in some cases conformed with the results reported by others in medicinal plants such as *Rosmarinus officinalis* (Mateu-andrés *et al*., 2013), *Mentha pulegium* L. (Ben Fadhel and Boussaïd 2004), *Teucrium polium L.*(Norouzi Ghare Tapeh *et al*, 2018), and *Helicrysum leucocephalum* (Azizi *et al*., 2014), that proposed geographical range is not always a predictable pattern for distribution of populations. On the other hand, high similarities among clustering and geographical distribution were shown in *Thymus caramanicus* (Hadian *et al*., 2014), *Teucrium arduini* L. (Kremer *et al*., 2015), *Salvia lavandulifolia* (Herraiz-Peñalver *et al*., 2017) and *Hypericum perforatum* L. (Morshedloo *et al*., 2014). In this study, it was obvious that the grouping based on morphological, phytochemical, and molecular traits were different, but there was a slight resemblance between morphological, phytochemical, and molecular clusters, such that Gajre and Polour ecotypes were clustered together in both morphological and iPBS clusters. Evard and Bajgiran and also Sorkhgarive and Ilam ecotypes were always grouped with each other in both phytochemical and iPBS clusters. Qazvin and Boroujen ecotypes were in the same group in all clustering, which showed the similarity of these two ecotypes for all aspects. Similar observations in *Satureja bachtiarica* (Khadivi-Khub *et al*., 2014), *Thymus* L. (Dalir and Safarnejad, 2017), *Origanum onites* L. (Tonk *et al*., 2010) and *Thymus caespititius* (Trindade *et al*., 2008) confirmed this aspect of the study that genetic similarities do not necessarily reflect similarities or differences in morphological or phytochemical traits.

CONCLUSIONS

Our results indicated ISSR and iPBS markers are useful for distinguishing and characterizing *Z.clinopodioides* ecotypes. We further emphasized that ISSR marker would be more suitable for analyzing the distinct genetic differences. The identification and selection of ecotypes that have the best breeding traits can be used for performing breeding work, therefore, Evard and Sorkhgarive ecotypes crosses with other populations can be a good option for producing hybrids with desired functions. For the protection of this important medical plant, successful cultivation could decrease

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the harvest of wild populations of *Z.clinopodioides*. Finally, it can be concluded that the molecular data may not have the expected alignment with morphological and phytochemical traits. This is not unexpected due to the influence of the environment on morphological and phytochemical traits.

More molecular and taxonomical investigations are required to better understand the differences between subspecies of this plant.

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ارزیابی فنوتیپی و ژنوتیپی برخی اکوتیپ های ایرانی کاکوتی کوهی (*Ziziphora clinopodioides* Lam.)

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چکیده

کاکوتی کوهی، یک گیاه دارویی از خانواده نعنائیان است که در سرتاسر ایران رشد می کند. در این مطالعه تنوع ژنتیکی ۱۰ اکوتیپ کاکوتی کوهی با استفاده از مارکرهای مورفولوژیکی، فیتوشیمیایی و مولکولی بررسی شد. مارکرهای ISSR و iPBS برای آنالیز مولکولی مورد استفاده قرار گرفت. میانگین باندهای چندشکل در هر پرایمر در مارکرهای ISSR و iPBS به ترتیب ۴/۴ و ۶/۶۸ بود و میانگین درصد پلی مورفیسم برای این مارکرها به ترتیب ۷۹/۴۹ ٪ و ۹۲/۰۳ ٪ بود. اکوتیپ های اوارد و سرخ گریوه بالاترین میزان صفات مهم اصلاحی را داشتند که شامل کوتاه ترین میانگره، بیشترین درصد وزن برگ به اندام رویشی، بیشترین عملکرد اسانس و محتوای فنل کل بود. نتایج نشان داد که اکوتیپ ها بیشترین تنوع را در همه ی مارکرهای مورد مطالعه داشتند. آنالیز تجزیه به مولفه های هماهنگ اصلی مطابق گروه بندی مولکولی بود اما داده های مورفولوژی و فیتوشیمیایی با این آنالیز همسو نبودند. علاوه بر این، هیچ همبستگی ای بین موقعیت جغرافیایی محل رشد گیاهان و داده های مارکرهای فیتوشیمیایی، مورفولوژیکی و مولکولی نبود.

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