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Population genetic and phylogeographic analyses of *Ziziphora clinopodioides* Lam., (Lamiaceae), "*kakuti-e kuhi*": An attempt to delimit its subspecies

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Abstract. *Ziziphora clinopodioides* Lam., (Lamiaceae), is a perennial herb which is used as traditional medicine in Iran. Different authors disagree on the number of subspecies. In general, taxonomic and biosystematic studies of *Ziziphora clinopodioides* have been limited and no molecular phylogenetic or biogeographic study of the species has been carried out. Therefore, the aims of this study were (1) to determine the number of subspecies, (2) to produce information on the species' genetic structure and intra-specific genetic variability, and (3) to produce data on the probable date of appearance of *Ziziphora clinopodioides* in Iran. We used a combination of morphological and molecular data to study plants randomly collected from 5 geographical regions. Both analyses revealed a high level of within population variability and grouping of the studied provinces produced an admixture that indicated the absence of any subspecies within the species. STRUCTURE analysis and K-Means clustering identified two gene pools within the country. The probable date of divergence obtained was 5-10 Mya for the appearance of this species in the mountainous regions of Qazvin and Mazandaran.

Keywords: biogeography, genetic diversity, STRUCTURE analysis, subspecies delimitation, Ziziphora clinopodioides.

INTRODUCTION

Species and subspecies delimitation is a difficult and somewhat subjective task in a species complex and in species with several overlapping geographical populations (Wiens 2007). In general, the recognition of a species or subspecies is based on morphological observations.

Different species can be delimited by a few distinct morphological characteristics that show no overlap with other species. This criterion is very tra-

ditional yet it makes sense biologically, which suggests that there is no gene flow between the species (based on some assumptions; for example any morphological difference has a genetic basis) (Wiens 2007). However, this approach can either fail to discriminate species and mask the presence of cryptic species or discriminate different species while in reality there is only one. In these situations, it is suggested that different and combined approaches such as morphological, molecular, cytological, and other approaches are used to determine species boundaries (Duminil and Di Michele 2009; Carstens *et al.* 2013). In some cases, incongruence may occur across the results from different methods. This may be due to either introgression or a difference in the power to detect cryptic lineages across one or more of the approaches used (Carstens *et al.* 2013).

In recent years, parallel developments in the analyzing power of both molecular phylogenetic and population genetic methods as well as their use in combination have resulted in more powerful species delimitation strategies. One of the most striking examples of a joint population genetics and phylogenetic approach is the use of the multispecies coalescent model to estimate phylogeny (Edwards 2009; Kingman 1982). This is further strengthened by development of new algorithms for detecting population genetic structure (Pritchard *et al.* 2000; Huelsenbeck and Andolfatto 2007; Huelsenbeck *et al.* 2011).

The procedure usually involves comparing clusters obtained on the basis of observed polymorphism in both morphological and molecular characters to test if they are in agreement. In case of infra-specific taxon identification (e.g. subspecies), the occurrence of discontinuity in both datasets can be suggestive (Seif *et al.* 2012; Koohdar *et al.* 2015).

Knowles and Carstens (2007) addressed how molecular data (i.e., gene trees from DNA sequence data) can be used in species delimitation. They proposed a new method which uses coalescent simulations to test hypotheses about species limits. Their method is particularly valuable in that it can incorporate data from multiple loci and does not require species to have diverged to the point of being reciprocally monophyletic. Similarly, Medrano *et al.* (2014), applied population genetics methods to the species delimitation problem in *Narcissus* (Amaryllidaceae) using amplified fragment length polymorphism (AFLP) molecular markers.

Ziziphora clinopodioides Lamarck (Lamiaceae) is a perennial herb with the common Persian name "*kakutie kuhi*". It is used as a traditional medicine in Iran to treat diseases such as the common cold, gastrointestinal disorders and inflammation (Naghibi *et al.* 2010). Controversy exists as to the number of subspecies that should be recognized. For example, there are nine subspecies native to Iran according to Flora Iranica (Rechinger 1982), but in the Flora of Iran (Jamzad 2012), no subspecies are considered.

Ziziphora clinopodioides has prostrated to erect stems and mainly branches at the base. The leaves vary in size and shape. The flowers are light to dark purple and white, with or without a peduncle, gathered in a compact capitulum. It is distributed in the eastern Balkan Peninsula, south east Asia and central Asia to the Pamir-Altay mountains and the Himalayas (Iran, Iraq and central and eastern parts of Turkey) as well as in Africa (Beikmohammadi 2011). In Iran it grows on rocky slopes, low hills and grasslands.

In general, there has been no detailed study looking at the taxonomy, molecular phylogeny and biogeography of this species. Therefore, the aims of this study were (1) to determine the number of subspecies, (2) to produce information on the species genetic structure and intraspecific genetic variability and (3) to produce data on the probable date of appearance in Iran of *Z. clinopodioides* and its ancestral area of distribution.

MATERIAL AND METHODS

Plant materials

In the present study, 69 plant specimens from 19 populations of *Z. clinopodioides* were randomly collected from five geographical localities (five provinces) of Iran. These populations occur from northern to eastern parts of the country and have almost a continous pattern of distribution. (Table 1, Fig. 1). Voucher specimens are deposited in the Herbarium of Shahid Beheshti University (HSBU).

Morphological studies

In total 29 morphological (5 qualitative, 24 quantitative) characters were studied. These characters include both vegetative and reproductive (floral) variables (Tables 2, 3).

Molecular studies

For molecular analyses, we used both multilocus molecular markers of inter-simple sequence repeats (ISSRs) as well as the chloroplast *rpL16* region. For ISSR analysis we used 69 specimens (1-6 samples from each

Table 1. Locality information for populations of *Z. clinopodioides* sampled, including herbarium vouchers for specimens used for morphological and ISSR analyses and GenBank Accession numbers for specimens used for cp-DNA analysis.

Pop no.	Province	Elevation (m)	Longitude	Latitude	Number of specimens sampled		Voucher No.	GenBank Accession no.
					ISSR & Morphology	cpDNA		
1	Razavi Khorasan	2042	352715.9	595344.9	4	1	HSBU2014413	(1) MG738475
2	Razavi Khorasan	1976	353559.4	5839.7	4	2	HSBU2014414	(2) MG738476
							HSBU2014427	(3) MG738477
3	Mazandaran	1039	363610.3	534952.7	4	3	HSBU2014415	(4) MG738478
							HSBU2014428	(10) MG738484
							HSBU2014429	(11) MG738485
4	Mazandaran	2597	368309	511855	6	2	HSBU2014421	(5) MG738479
							HSBU2014430	(6) MG738480
5	Tehran	2978	354349	521384.8	4	1	HSBU2014425	(7) MG738481
6	Qazvin	1400	362765.5	501711.4	5	2	HSBU2014431	(8) MG738482
							HSBU2014432	(9) MG738483
7	Mazandaran	2225	363107	5456	3	2	HSBU2014426	(12) MG738486
							HSBU2014433	(13) MG738487
8	Ardebil	1493	381209.9	483909.2	6	1	AUH522	(14) MG738488
9	Ardebil	1389	381235	481757.3	3	1	ALUH526	(15) MG738489
10	Tehran	2308	355775.2	512954.5	2		HSBU2014424	
11	Qazvin	2750	392936	573450	4		HSBU2014416	
12	Qazvin	1333	363155.1	509824	5		HSBU2014417	
13	Razavi Khorasan	2652	362319.7	5959.5	6		HSBU2014412	
14	Mazandaran	2103	362633.5	512838	1		HSBU2014419	
15	Mazandaran	2299	355514	521172.8	2		HSBU2014420	
16	Mazandaran	2341	355293.4	528320	4		HSBU2014423	
17	Mazandaran	1510	311137.1	523006.6	1		AUH529	
18	Tehran	3245	362229.8	512628.2	3		HSBU2014422	
19	Tehran	2398	354636.4	515869.6	2		HSBU2014418	

population) and for cpDNA analysis, we used a subset of 15 randomly selected plants (1-3 samples) from the studied populations of five provinces (Table 1).

Both markers are widely used for species diversity analysis and phylogeny (Weising *et al.* 2005; Sheidai *et al.* 2014). ISSRs are particularly suitable markers for infra-specific studies and can reveal genetic discontinuities among populations (Sheidai *et al.* 2012; Sheidai *et al.* 2013).

DNA extraction, amplification and ISSR assay

Genomic DNA was extracted using a CTAB (cetyl trimethyl-ammonium bromide) activated charcoal protocol (Sheidai *et al.* 2013). The quality of extracted DNA was examined by running on a 0.8% agarose gel.

10 ISSR (inter simple sequence repeat) primers, $(AGC)_5GT$, $(CA)_7GT$, $(AGC)_5GG$, UBC810, $(CA)_7AT$, $(GA)_9T$, UBC807, UBC811, $(GA)_9A$ and $(GT)_7CA$, were used (University of British Columbia). PCR reactions were performed in a 25µl volume containing 10 mMTris-HCl buffer at pH 8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Bioron, Germany), 0.2 µM of each primer, 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The reactions were performed in a Techne thermocycler (Germany) with the following program: 5 min initial denaturation step at 94 °C, followed by 40 cycles of 45s at 94 °C; 1 min at 60 °C and 1min at 72 °C. The reaction was completed with a 7 min extension step at 72 °C.

The amplification products were visualized by running on 2% agarose gels. The fragment size was estimated using a 100 bp molecular size ladder (Fermentas, Germany). In order to identify reproducible bands, the experiment was replicated 3 times.

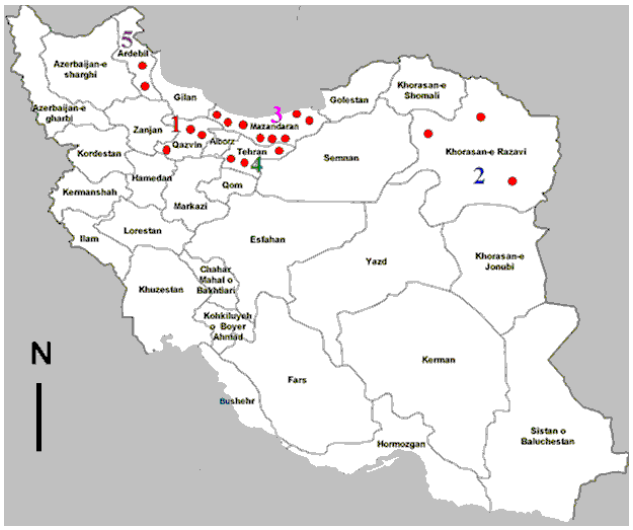

Figure 1. Distribution map of the studied provinces.

Chloroplast DNA

The intron in the gene for ribosomal protein L16 (*rpL16*) located in the chloroplast genome was amplified and sequenced with universal primers following the methodology of Shaw and Small (2005) and Timmer *et al.* (2007). The *rpL16* forward primer was 5′-GTAAGGGTCATTTAGTAGGTCGTTT -3′ and the reverse primer 5′-TCCTTACCATTAAGTTGATC -3′. Each 20 µl PCR tube contained 10 µl of 2x PCR buffer, 0.5 mM of each primer, 200 mM of each dNTP, 1 Unit of *Taq* DNA polymerase (Bioron, Germany), and 1 µl of template genomic DNA at 20 ng μl^{-1}. The amplification reaction was performed in a Techne thermocycler (Germany) with the following program: 2 min initial denaturation step at 94°C, followed by 35 cycles of 5 min at 94°C; 1.30 min at 62°C and 2 min at 72°C. The reaction was completed by a final extension step of 7 min at 72°C.

PCR products were visualized on 2.5% agarose gels with GelRed™ Nucleic Acid Gel Staining. Fragment sizes were estimated using a 100 bp size ladder (ThermoFisher Scientific, Waltham, MA USA).

Data analyses

Morphometry

Morphological characters were first standardized (Mean = 0, Variance = 1) and used to establish Euclidean distances among pairs of taxa (Podani 2000). For grouping of the plant specimens, the UPGMA (Unweighted pair Group Method with Arithmetic Mean) and ordination method of PCA (principal components analysis) were used (Podani 2000). A PCA (principal components analysis) biplot was used to identify the most variable morphological characters among the studied populations (Podani 2000). PAST version 2.17 (Hammer *et al.* 2012) was used for multivariate statistical analyses of morphological data.

Table 2. Qualitative morphological characters studied in *Z. clinopodioides* populations.

Character	State of character and their codes
Vegetative form	Straight (1), Geniculate (2)
Basal vegetative form	Woody (1), Dense woody (2), Sparse woody-stacked (3)
Stem leaf shape	Lanceolate (1), Lanceolate-ovate (2), Multiform (3)
Calyx hair frequency	Frequent (1), Sparse (2), Very sparse (3)
Calyx pedicle	Present (1), Not present (2)

Table 3. Quantitative morphological characters studied in *Z. clinopodioides* populations.

No.	Characters
1	Plant length (cm)
2	Leaf length of stem(mm)
3	Leaf width of stem(mm)
4	Stem Leaf length / width ratio
5	Petiole length(mm)
6	Inflorescence leaf length (mm)
7	Inflorescence leaf width (mm)
8	Inflorescence leaf length/ width ratio
9	Pedicle length (mm)
10	Calyx length(mm)
11	Calyx width(mm)
12	Calyx length/ width ratio
13	Calyx teeth length(mm)
14	Calyx teeth width(mm)
15	Calyx teeth length/ width ratio
16	Inflorescence length(cm)
17	Inflorescence width(cm)
18	Inflorescence length/ width ratio
19	Corolla length(mm)
20	Corolla tube length(mm)
21	Petal length(mm)
22	Corolla tube length/Petal length
23	Stamen length(mm)
24	Style length(mm)

ISSR analyses

ISSR bands obtained were coded as binary characters (presence = 1, absence = 0). For grouping of the studied provinces, ISSR bands obtained were coded as binary characters (presence = 1, absence = 0). For grouping of the studied provinces, PCO plot (principle coordinate analyses) was used (Noormohammadi *et al.* 2011).

The Mantel test was performed to check correlation between geographical distance and genetic distance of the studied provinces (Podani 2000). The PAST ver. 2.17 (Hammer *et al.* 2012) program was used for these analyses.

AMOVA (Analysis of molecular variance) based on Fst and Nei's Gst as implemented in GenAlex 6.4 (Peakall and Smouse 2006) was used to reveal genetic difference of the studied provinces. In order to determine the genetic structure of geographical provinces, we used two different approaches. First, Bayesian model based STRUCTURE analysis (Pritchard *et al.* 2000), and second, the maximum likelihood based method of K-means clustering. For STRUCTURE analysis with 10^5 permutations, data were scored as dominant markers (Falush *et al.* 2007). We performed K-means clustering in GenoDive ver. 2. (2013). Two summary statistics, 1) pseudo-F, and 2) the Bayesian Information Criterion (BIC), provide the best fit for k in the K-Means clustering method (Meirmans 2012).

The population assignment test was performed using the maximum likelihood method as implemented in GenoDive (Meirmans and Van Tienderen 2004).

In order to identify agreement between the genetic tree and the morphological tree, we obtained a consensus tree using DARwin ver.5 (2012).

cp-DNA sequence analyses and estimation time of divergence

The intron in the gene for ribosomal protein L16 (*rpL16*) was aligned with MUSCLE (Robert, 2004) implemented in MEGA 5. The molecular clock test was performed as implemented in MEGA 5 (Tamura *et al.* 2011). The test was done by comparing the ML value for the given topology with and without the molecular clock constraints under the Tamura and Nei (1993) model. using the parsimony method of Templeton *et al.* (1992), implemented in TCS 1.13 program (Clement *et al.* 2000). Before estimating time of divergence, we used MEGA 5 to test the molecular clock and to find the best substitution model for the given sequences. The equal evolutionary rate of the studied sequences was rejected at a 5% significance level and therefore we used the relaxed molecular clock model in further analyses (Drummond *et al.* 2006). Moreover, HKY was the best substitution model identified by model test as implemented in MEGA 5 (Posada and Crandall 1998).

BEAST v1.6.1 (Drummond *et al.* 2010a; Drummond *et al.* 2010b) was used for the Bayesian MCMC inferred analyses of the nucleotide sequence data (Drummond and Rambaut 2007). *Lallemantia baldschuanica* Gontscharow, *L. iberica* Fisch. & C.A. Mey. and *L. royleana* Bentham were used as outgroups.

BEAUti (Bayesian Evolutionary Analysis Utility version) v1.6.1 (Drummond *et al.* 2010a, 2010b) was utilized to generate initial xml files for BEAST. A Yule process of speciation (a 'pure birth' process) was used as a tree prior for all the tree model analyses.

The Yule tree prior is widely recognized as giving the best-fit model for trees describing the relationships between different species (Drummond *et al.* 2010a, 2010b) and can be regarded as explaining the net speciation rate (Nee 2006). For the MCMC analyses, the chain length was 10000000. After discarding 100 trees representing the burn-in, 10000 trees were used for the analyses. The BEAUti xml file was run in BEAST v1.6.1 (Drummond *et al.* 2010a, 2010b). Because no fossils are available for the studied species, we assumed a rate of evolution of the plastid sequence ($u = 1.0 \times 10^{-9}$ s s^{-1} $year^{-1}$) (Zurawski *et al.* 1984; Minaeifar *et al.* 2016). This was included in the option of molecular clock model in BEAUti v1.6.1. The normal distribution (Mean = 0, Standard deviation = 1) was used for priors.

Tracer v1.5 (Drummond and Rambaut 2007) was used to examine sampling and convergence. Tree Annotator v1.6.1 (Drummond and Rambaut 2007) was used to annotate the phylogenetic results generated by BEAST to form a single 'target' tree (Maximum Clade Credibility tree, MCC) including summary statistics. FigTree v1.3.1 (Rambaut 2009) was used to produce the annotated BEAST MCC tree (Fig. 6).

Biogeography

The distribution range of *Ziziphora clinopodioides* studied was divided into 5 areas (provinces): A (Razavi Khorasan), B (Ardebil), C (Mazandaran), D (Qazvin) and E (Tehran). We used S-DIVA (Statistical Dispersal-Vicariance Analysis) and BBM (Bayesian Binary Method) analyses implemented in RASP to reconstruct the possible ancestral ranges on the phylogenetic trees (Yu *et al.* 2010, Yu *et al.* 2015). In these methods, the frequencies of an ancestral range at a node in ancestral reconstructions are averaged over all trees (Yan *et al.* 2010). We used initially the tree obtained from the BEAST

analysis (MCC tree), followed by RASP analysis. The final tree for the area ancestry determination was based on the majority rule consensus tree.

RESULTS

Systematics

Morphometry

The mean values and standard errors for the quantitative morphological characters are provided in Table 4.

The ANOVA test revealed significant difference in stem leaf length ($p = 0.01$), inflorescence leaf length / width ratio ($p = 0.01$) and corolla tube length/petal length ratio ($p = 0.02$).

Different clustering and ordination methods produced similar results, therefore only the PCA plot of the studied provinces based on the morphological data is provided (Fig. 2). The studied provinces were placed inter-mixed, thus there is no support for morphological divergence among provinces.

There appears to be some morphological differentiation between province 2 (Razavi Khorasan) and all other provinces which is plausible as it is the most geographically separated (Fig. 2).

PCA analysis of morphological characters revealed that the first three PCA components comprised 70% of the total variability. Morphological traits (stem leaf shape, petiole length and style length) showed the highest level of correlation with the first PCA component (>0.65), while characters 6 and 7 were highly correlated with the second PCA component (>0.62). Therefore, these are the most variable morphological characters among the five studied provinces. The PCA biplot, (not shown) revealed that morphological characters 3 and 10 differentiate mainly province 2 (Razavi Khorasan), while character 26 differentiates province 5 (Ardebil) from the others.

ISSR analysis

ISSR analysis of the studied provinces produced 97 reproducible bands. The PCO plot (Fig. 3) revealed that plants from different provinces were grouped together due to genetic similarity, for example those from provinces 2, 3 and 4. Therefore, ISSR data do not differentiate the studied provinces. This is in agreement with our morphometric analyses.

Table 4. The mean value and standard error of quantitative morphological characters.

Character	Qazvin	Razavi Khorasan	Mazandaran	Tehran	Ardebil
	14 specimens	14 specimens	21 specimens	11 specimens	9 specimens
Leaf length of stem(mm)	15.50 ±0.73	8.00 ±0.49	12.30 ±1.16	15.18 ±0.74	10.07 ±0.36
Leaf width of stem(mm)	4.50 ±2.00	3.42 ±0.27	3.60 ±0.23	3.80 ±0.35	4.00 ±0.44
Stem Leaf length / width ratio	3.48 ±0.16	2.41 ±0.13	3.32 ±0.12	4.18 ±0.28	2.90 ±0.26
Petiole length(mm)	1.75 ±0.23	2.85 ±0.77	1.60 ±0.13	1.40 ±0.13	1.73 ±0.87
Inflorescence leaf length (mm)	7.04 ±1.03	4.94 ±0.44	6.74 ±0.62	6.77 ±0.84	7.35 ±0.63
Inflorescence leaf width (mm)	2.40 ±0.20	2.50 ±0.20	3.00 ±0.22	2.52 ±0.31	3.25 ±0.27
Inflorescence leaf length/ width ratio leaf width	3.31 ±0.31	2.50 ±0.11	2.23 ±0.11	2.80±0.21	2.30 ±0.31
Pedicle length (mm)	1.33 ±0.13	0.49 ±0.10	1.40 ±0.07	1.38 ±0.06	1.51 ±0.07
Calyx length(mm)	4.07 ±0.13	4.58 ±0.27	5.24 ±0.15	8.25 ±3.48	5.17 ±0.18
Calyx width(mm)	1.05 ±0.08	1.15 ±0.10	1.20 ±0.05	1.25 ±0.08	1.25 ±0.02
Calyx length/ width ratio	4.08 ±0.30	4.29 ±0.22	4.41 ±0.15	3.83 ±0.17	4.11 ±0.13
Calyx teeth length(mm)	0.78 ±0.07	0.96 ±0.06	0.93 ±0.06	0.81 ±0.09	0.82 ±0.05
Inflorescence length(cm)	1.63 ±0.06	1.33 ±0.09	1.37 ±0.08	1.29 ±0.13	1.45 ±0.17
Inflorescence width(cm)	1.84 ±0.05	1.82 ±0.06	1.62 ±0.07	1.59 ±0.09	1.53 ±0.14
Inflorescence length/ width ratio	0.86 ±0.03	0.82 ±0.05	0.83 ±0.03	0.83 ±0.08	0.94 ±0.05
Corolla length(mm)	5.87 ±0.27	6.21 ±0.33	6.28 ±0.27	6.12 ±0.26	6.21 ±0.45
Corolla tube length(mm)	3.48 ±0.16	3.57 ±0.19	3.80 ±0.18	3.43 ±0.17	4.03 ±0.38
Petal length(mm)	2.25 ±0.17	2.60 ±0.20	2.48 ±0.12	2.69 ±0.12	2.18 ±0.09
Corolla tube length/Petal length	1.54 ±0.10	1.48 ±0.13	1.56 ±0.08	1.26 ±0.06	1.84 ±0.13
Stamen length(mm)	1.19 ±0.16	2.07 ±0.28	1.78 ±0.22	0.68 ±0.21	1.99 ±0.26
Style length(mm)	4.78 ±0.23	4.81 ±0.41	5.13 ±0.28	4.59 ±0.28	4.54 ±0.29

Mean ± standard error.

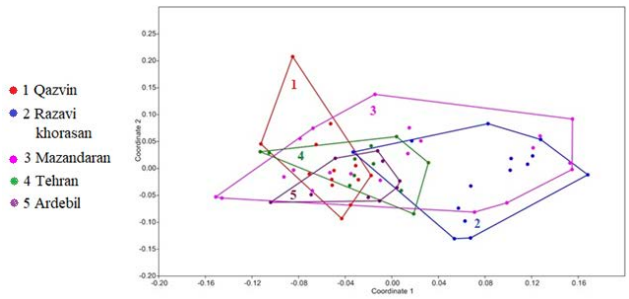

Figure 2. PCA plot of *Ziziphora clinopodioides* provinces based on 29 morphological characters.

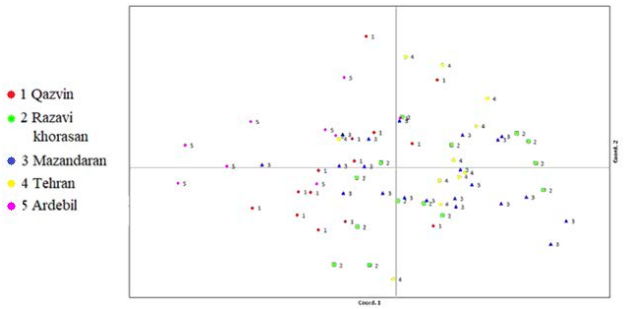

Figure 3. PCO plot of *Ziziphora clinopodioides* provinces based on ISSR data.

Moreover, the consensus tree of morphological and genetic features did not differentiate the plants collected in the studied provinces (Fig. not given), only distinguishing plant numbers 6 and 7 of Qazvin province (Province 1), and plants 46 and 47 of Mazandaran province (Province 3). This result suggests that morphological variation in the studied provinces is not in agreement with their genetic features. Therefore, the present study does not support the idea that *Z. clinopodioides* contains any subspecies in Iran. This conclusion is further supported by haplotype networking of cp-DNA (Fig. 4).

The studied plants differed in cp-DNA sequences. The haplotype network separated outgroups from the studied *Ziziphora clinopodioides* plants. Moreover, it revealed large-scale within-province cp-DNA variation. For example, plants studied in Mazandaran, Ardebil and Razavi-Khorasan provinces were widely scattered on the network.

Provincial genetic diversity analyses

Genetic diversity parameters from the studied provinces are presented in Table 5. The highest value of genetic polymorphism in province 3 (79.38%) and the

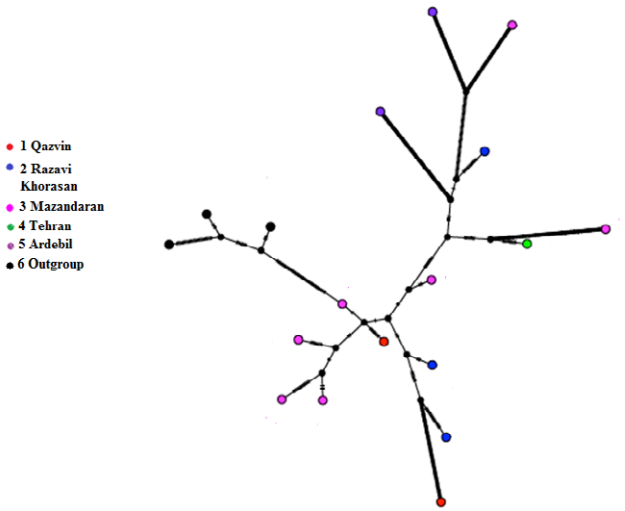

Fig 4. Haplotype network of cp-DNA data in the studied *Ziziphora clinopodioides* provinces.

highest value of Nei gene diversity occurred in province 2 (0.158), while the lowest value of the same parameters was observed in province 5 (45.36% and 0.123, respectively). This indicates that province 5 has a lower degree of within province genetic variability.

AMOVA and Gst results revealed significant difference among the studied provinces. AMOVA produced a PhiPT value of 0.068 (P = 0.01), while the Gst value was 0.065 (P = 0.01). Pair-wise analysis of Fst and Gst revealed significant difference between provinces (Table 6).

AMOVA revealed that 93.2% of total genetic variability occurred due to within province diversity and 6.87% due to among province diversity. This is in agreement with PCO plot of ISSR data presented before; the provinces were not differentiated.

Migration analysis of genetic data in all populations of five provinces produced a mean Nm value of 6.45 and

Table 5. Genetic diversity parameters in the studied provinces based on ISSR data

Province	N	Na	Ne	I	He	UHe	%P	Hs
Qazvin	14	1.340	1.191	0.227	0.134	0.139	67.01	0.218
Razavi Khorasan	14	1.464	1.227	0.262	0.158	0.163	73.20	0.251
Mazandaran	21	1.588	1.193	0.246	0.142	0.145	79.38	0.234
Tehran	11	1.361	1.197	0.242	0.143	0.149	68.04	0.242
Ardebil	9	0.907	1.192	0.195	0.123	0.130	45.36	0.185

N = No. plants, Na = No. alleles, Ne = No. effective alleles, I = Shanon Information Index,
He = Nei gene diversity, UHe = Unbiased gene diversity, %P = Percentage of genetic polymorphism, and Hs = Genetic diversity due to population.

Table 6. Pair-wise analysis of Fst in the studied provinces based on ISSR data.

	Qazvin	Razavi Khorasan	Mazanda-ran	Tehran	Ardebil
Qazvin	0.000				
Razavi Khorasan	0.075	0.000			
Mazandaran	0.035	0.030	0.000		
Tehran	0.057	0.054	0.028	0.000	
Ardebil	0.099	0.156	0.123	0.132	0.000

a Gst value of 0.07. These values indicate a high degree of gene flow among the studied populations. Moreover, STRUCTURE analysis based on a genetic admixture model also revealed a high degree of genetic admixture among the studied provinces as they had very similar allele combinations (similarly colored segments). These common shared alleles are either ancestral shared alleles or occurred due to ongoing gene flow among the populations. The Evanno test identified two gene pools.

The province assignment test revealed that gene flow occurred between all provinces but was higher between plants in provinces 1, 3 and 4. Province 5 had the lowest degree of gene flow as revealed by the lowest within province genetic variability as stated above. This province had limited gene flow with provinces 3 and 4.

The pseudo-F value of K-Means clustering and Evanno test of STRUCTURE revealed two genetic groups. When we performed the STRUCTURE analysis for k = 2 (Fig. 5), it revealed that provinces 1 and 5 formed the first genetic group, while provinces 2-4 comprised the second genetic group. Therefore, we have two gene pools in Iran for this medicinal plant that can be used in germplasm conservation and future medicinal evaluation.

The Mantel test produced significant correlation (r = 0.184, *P* = 0.01) between geographical distance and genetic distance of the studied provinces. This means that IBD (Isolation by distance) has occurred in *Z. clinopodioides* provinces and the neighboring provinces can

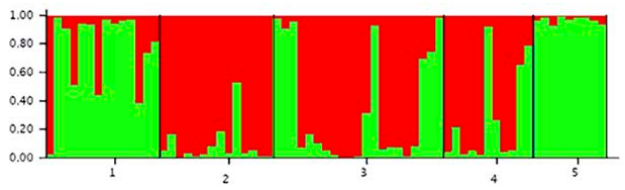

Figure 5. STRUCTURE plot of *Ziziphora clinopodioides* provinces based on k = 2. (Provinces 1-5 are: 1- Qazvin, 2- Razavi Khorasan, 3- Mazandaran, 4- Tehran, and 5- Ardebil).

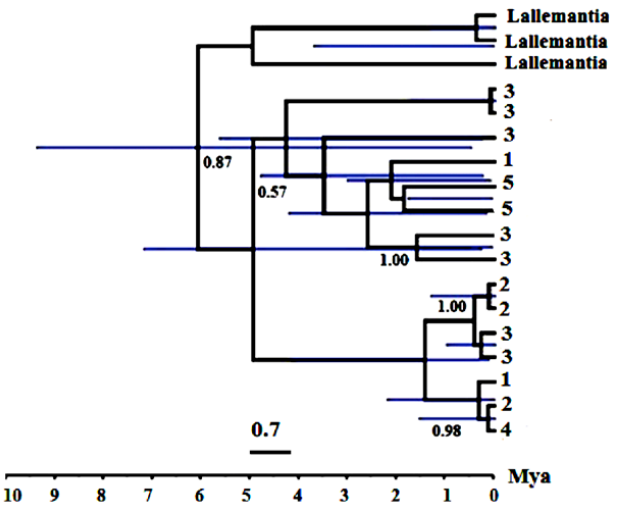

Figure 6. Chronogram from BEAST analysis of the studied provinces for *Ziziphora clinopodioides* based on the cp-DNA dataset (*rpL16*), showing 95% highest posterior density bars (HPD) in purple. Numbers on nodes are clade credibility values. (Provinces 1-5 are: 1- Qazvin, 2- Razavi Khorasan, 3- Mazandaran, 4- Tehran, and 5- Ardebil).

exchange genes more frequently compared to those that are further from each other. This could be the reason for the higher degree of genetic similarity observed between provinces 2, 3 and 4.

Divergence time estimation

cp-DNA haplotypes can be considered as good molecular markers for investigating probable dates of appearance of populations and their paths of distribution in the country (Minaeifar *et al*, 2016). BEAST and RASP analyses (Figs 6, 7) suggested that the oldest cp-DNA haplotype of *Z. clinopodioides* appeared sometime around 5-10 Mya in Mazandaran province (province 3). This suggests that *Z. clinopodioides* could possibly have appeared in the northern regions of the country during the Miocene era, with plants subsequently dispersing towards the north-eastern (Razavi Khorasan), north-western (Ardebil) and central parts of Iran (Tehran and Qazvin).

DISCUSSION

In the present study molecular markers such multilocus ISSRs and cp-DNA sequences and morphological variables were used for genetic diversity, species and subspecies delimitation of Z. *clinopodioides*. According

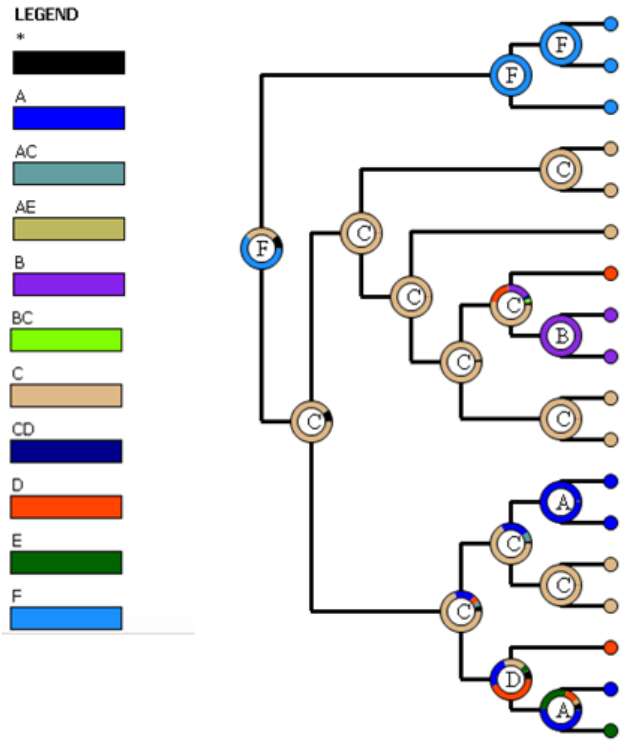

Fig 7. Cp-DNA RASP analysis based on BEAST tree (MCMC) showing probable ancestral area distribution for *Ziziphora clinopodioides* provinces. Razavi Khorasan, B- Ardebil, C- Mazandaran, D- Qazvin, E- Tehran and F- *Lellemanthia* (outgroup).

to several evaluations, phylogenetic markers (ITS and cpDNA) and ISSR molecular techniques are useful for genetic diversity, species and subspecies delimitation for different taxa such as, *Diospyros* L. (Li *et al.* 2018), *Marrubium* L. (Salehi *et al.* 2018), *Carum* L. (Papini *et al.* 2015), *Acer velutinum* Boiss (Siahkolaee 2017), *Cycas diannanensis* Z. T. Guan & G. D. Tao (Jian *et al.* 2015) and *Petunia axillaris* (Lam.) Britton, Sterns & Poggenb (Turchetto *et al.* 2014).

Both molecular markers (multilocus ISSRs as well as cp-DNA sequences) and morphological characters produced similar results and showed a lack of province discontinuity within *Z. clinopodioides*. Therefore, our data do not suggest the presence of subspecies in the studied populations of this species. Jamzad (2012) in the Flora of Iran, after thorough morphological investigation in *Z. clinopodioides*, suggested that due to a high degree of morphological variability and co-occurrence of many subspecies in one location, she could not be sure about the number of subspecies within *Z. clinopodioides* and suggested the use of molecular studies to solve this problem.

Moreover, high morphological, palynological and molecular diversity exist among *Ziziphora* taxa (Tabaripour *et al.* 2018; Tabaripour *et al.* 2019) and the genus shows very variable chromosome number along a descending dysploidy line starting from 2n = 16 to 2n = 34 (Taarna 1973; Selvi *et al.* 2013), but *Z. clinopodioides* proved to has 2n=18 (Selvi *et al.* 2013).

In a similar study, subspecies determination was conducted in the Western Australian species *Pityrodia scabra* A.S. George. (Lamiaceae) using a combined approach with non-coding chloroplast gene regions and morphological data (Shepherd *et al.* 2013). They observed that some morphological features varied among the populations and provided some evidence for cryptic taxa. Furthermore, molecular phylogenetic analyses revealed genetic distinctiveness between the Wyalkatchem (type) population and the Southern Cross and Lake Lefroy populations. This evidence, when used in conjunction with the morphological differences, provided support for the recognition of the new subspecies described as *Pityrodia scabra* subsp. *dendrotricha* K.A. Sheph. subsp. nov.

Population genetics studies are an important step in planning genetic and breeding programs for crop and medicinal plants. They provide data on genetic variability, gene flow versus population genetic isolation, population genetic fragmentation, alongside the role of genetic drift, bottlenecks and other evolutionary forces acting on population divergence (Sheidai *et al.* 2013, 2014).

With increases in sizes of human populations, crop plants and medicinally important plant taxa are consumed and destroyed faster than before. Medicinal plants such as *Z. clinopodioides* are extensively used by locals and therefore potentially threatened in their natural habitats. Therefore, to design an effective conservation strategy, knowledge of genetic diversity in the target species is important.

The present study revealed a high level of morphological and genetic variability both within and among provinces of *Z. clinopodioides*. AMOVA revealed that 93% of total genetic variability occurred due to within province diversity and 7% due to among province diversity. This could be due to the out-crossing nature of this species. These plants are usually cross pollinated in nature by insects, which can result in high within population genetic variability. We can exploit this variability in future hybridization and breeding strategies.

Assessments of levels of within- and among-population genetic variations have been used to prioritize populations for conservation efforts (Petit *et al.* 1998), with (all else being equal) more weight given to populations exhibiting higher levels of within-population variation and to those that are more genetically divergent.

The STRUCTURE plot and province assignment revealed some degree of genetic admixture among the

studied *Z. clinopodioides* provinces. Gene flow is also important in conservation contexts, particularly for species with local populations. Fortunately, *Z. clinopodioides* provinces showed high within-province genetic variability and high among province gene flow. Gene flow among local populations could mitigate losses of genetic variation caused by genetic drift in local populations and potentially save them from extinction (Sheidai *et al.* 2014; Safaei *et al.* 2016).

The Mantel test revealed isolation by distance in the studied *Z. clinopodioides* provinces. In plant species that form geographical populations, as geographical isolation increases, a reduction in both seed dispersal and pollen flow will result in decreased gene flow between distantly located populations (Freeland *et al.* 2011). This explains why the Evanno test and K-Means clustering identified two different gene pools for *Z. clinopodioides* within the country.

BEAST and RASP results suggested that *Z. clinopodioides* haplotypes appeared around 5-10 Mya in the mountainous regions of Qazvin and Mazandaran. Active divergence occurred between 1-5 Mya in these mountains due to their reactions to Pleistocene glaciations. Our mean date of 7 Mya is in agreement with the study of Drew and Sytsma (2012).

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