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Genetic divergence and speciation within *Ziziphora capitata* (Lamiaceae): Molecular and micromorphological evidences

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Abstract. *Tabaripour R, Sheidai M, Talebi SM, Noormohammadi Z. 2018. Genetic divergence and speciation within* Ziziphora capitata *(Lamiaceae): Molecular and micromorphological evidences. Biodiversitas 19: 747-755. Ziziphora capitata* is a member of the Mint family (Lamiaceae) that naturally grows in various regions. Different subspecies have been reported for this species, from which two are recorded from Iran: *Z. capitata* subsp. *capitata*, and *Z. capitata* subsp. *orientalis*. In the present study, morphological, palynological and molecular (ISSR and cp-DNA) data were used to reveal speciation within this species and reconsider taxonomic rank of its presumed subspecies. The obtained results revealed a great difference between two presumed subsp. of *Z. capitata*. These taxa differed in morphological, palynological and molecular characteristics. Statistical and bioinformatics tests revealed significant genomic difference between them and phylogenetic analyses showed that they have a close affinity to some other *Ziziphora* species (out-groups used), then to each other. Bayesian and maximum parsimony phylogenetic trees differentiated the presumed sub-species of *Z. capitata*. Moreover, K-Means clustering and Bayesian analysis of genetic groups by Evanno test (optimal k) supported the presence of two distinct genetic groups within *Z. capitata*. This approach objectively defines a threshold separating intraspecific population substructure from interspecific divergence, which is the general aim of species delimitation studies. Therefore, the present study provides enough evidence for introducing two separate species within *Z. capitata* based on the traditional taxonomy as well as combination of molecular and micro-morphological data.

Keywords: Bayesian tree, genetic distance, multiple approaches, species delimitation, *Ziziphora*

INTRODUCTION

The members of Labiatae are economically important and very famous for their usages as culinary herbs, horticultural plants as well as in traditional and modern medicine. Although, Lamiaceae is one of the largest plant families with high taxonomical complexity and its species are widely distributed worldwide, there are very few molecular phylogenetic analyses at infrageneric level (Trusty et al. 2004; Stevens et al. 2008).

The genus *Ziziphora* L. (Lamiaceae) consists of four species in Iran namely, *Z. clinopodioides* Lam., *Z. capitata* L., *Z. persica* Bunge. and *Z. tenuior* L. (Rechinger 1982; Mozaffarian 1996). *Z. capitata* is an annual herb that grows in Europe, Central Asia, Iraq, Syria, Turkey, Caucasus and Iran that is distributed from northern to southwestern parts (Jamzad 2012).

The aerial parts, leaves and flowers of some *Ziziphora* species have been generally used as medicinal plant in Iranian folk medicine for sedative, stomach tonic, flatulence, common cold, diarrhea, expectorant, coughing, antiseptic, migraine, fever and carminative (Zargari 1996; Nejad-Ebrahimi et al. 2009). The chemical composition of the hydrodistilled essential oil of the aerial flowering parts of *Z. capitata* from Iran analyzed by GC and GC-MS identified 42 components representing 97.2 % of the total oil.

Z. capitata has simple or branched stem and its length varies from 3 to 12 cm. The aerial parts of this plant are covered by glandular and non-glandular trichomes. The basal leaves are linear-lanceolate to elliptic with 1 to 2.5 cm long and up to 0.8 cm width. The floral leaves are in the shape rhombic-ovate. Inflorescence is capitate with rhombic-ovate bracts and consisted of tubular calyx with pink, violet and purple corolla (Brands 2017).

Based on the morphological data, two subspecies have been reported for *Z. capitata* in Iran: *Z. capitata* subsp. *capitata*, and *Z. capitata* subsp. *orientalis* (Rechinger 1982). In *Z. capitata* subsp. *capitata*, the size of floral leaf is relatively longer than the flowers, while, in *Z. capitata* subsp. *orientalis* it is smaller in size. The shape of floral leaf in *Z. capitata* subsp. *orientalis* is ovate to circular while in *Z. capitata* subsp. *capitata* it is lanceolate to linear. Similarly, the bract apex is obtuse in the first subsp., while it is acuminate in the second.

Molecular data have been used in phylogenetic studies and species divergence investigations (Sheidai et al. 2013 2014). These data can also provide supportive and additional criteria for systematic classification of the species studied that have been merely based on the morphological characters (Minaeifar et al. 2016), and produce evidence for identification of infra-specific taxonomic forms viz. subspecies and varieties (Nikzat-Siahkolaee et al. 2017).

Chloroplast DNA [cpDNA] restriction site analysis and nucleotide sequence data have been used in recognition and recircumscription of the Lamiaceae as a monophyletic assemblage (Olmstead and Palmer 1994, Salmaki et al. 2013). However, within Lamiaceae, the species relationships in some genera are still unresolved (Salmaki et al. 2013).

The present study is concerned with the molecular, morphological and micromorphological data of presumed subsp. within *Z. capitata* and tries to reconsider their taxonomic status.

Leavitt et al. (2015) have recently stated that each species shows a basal unit in evolutionary biology, which may be utilized for organizing as well as evaluating the biological concepts and also principles. With recent bioinformatical and methodological progressions, delimitation of empirical taxa become a dynamic science. Moreover, amplifying availability of nuclear, chloroplast and mitochondrial DNA data, new analytical approach, maximizing computational power, reassessments of different morphological, anatomical and also chemical features, exhibit mediums for showing taxa delimitation and also evolutionary relations among lineages of species-level.

Various approaches to species delimitation may yield different estimates of species boundaries, which in turn under influence of subjective interpretation of the results obtained. However, recent and more sophisticated approaches, include selection of species delimitation models using approximate Bayesian computing and designing and conducting a simulation study that matches the characteristics of the empirical study can be used to more objectively evaluate competing hypotheses of species boundaries (Leavitt et al. 2015). Objectively defining a threshold separating intraspecific population substructure from interspecific divergence is the aim of species delimitation studies. Most species delimitation methods based on single-locus sequence data fall under two general categories: either genetic distance or tree-based approaches (Sites and Marshall 2004). Therefore, in order to reach the most probable objective species limitation, here a multiple approach study is used for *Z. capitata*. Phylogenetic analyses (tree-based method) on both single locus and multilocus molecular markers, genetic distance estimation, morphological and micro-morphological analyses were performed to provide sufficient evidence for species delimitation and finally suggested upgrading the presumed subspecies to the species level.

MATERIALS AND METHODS

Plant materials

The sampling locations of *Z. capitata* subspecies, and outgroups studied (*Z. tenuior*, *Z. persica* and *Z. clinopodioides*) were selected from provided addresses by flora Iranica (Rechinger, 1982) and flora of Iran (Jamzad 2012) (Table 1). The identification of taxa studied was based on the descriptions on the references. Five plant specimens from each subspecies of *Z. capitata* were used for morphology, palynology and Molecular (ISSR) studies.

Morphometry studies

In morphological investigations twenty-five qualitative and quantitative variables of both vegetative and reproductive organs were studied (Tables 2 and 3).

Table 1. Localities addresses of the *Ziziphora* taxa studied

Taxa	Locality	Voucher number
Z. tenuior	North Khorasan, 45 km from Bojnord, 2042 m.	T 26342
Z. clinopodioides	Qazvin, Alamut-e Gharbi,Roudbar Qastin Lar, 1350-1400 m.	HSBU2014431
Z. persica	West Azerbaijan, Urmia,1656 m.	HSBU2014434
Z. capitata subsp. *orientalis*	Alborz, Shahrestanak, 1452 m	HSBU2014436
Z. capitata subsp. *orientalis*	East Azarbaijan, Tabriz, 1700 m.	HSBU2014437
Z. capitata subsp. *capitata*	East Azarbaijan, Ghaleh-e-babak,1400-1500 m.	TUM 42703
Z. capitata subsp. *capitata*	Lorestan, road of Aleshtar, 2250 m.	TUM 21688

Table 2. The qualitative morphological characters in *Ziziphora* species studied

Character	State of character and their codes
Lifeform	Annual (1), perennial (2)
Floral type	Spiciform (1), capitate (2)
Floral shape	Oblong-narrow (1), ovate (2), orbicular (3)
Floral leaf	Longer than inflorescence (1), as long as or shorter than inflorescence (2)
Floral leaf shape	Wide ovate (1), lanceolate (2) lanceolate-ovate (3), orbicular (4), obtuse (1), acuminate (2)
Bract apex	

Table 3. The quantitative morphological characters in *Ziziphora* species studied

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	Floral leaf length (mm)
6	Floral leaf width (mm)
7	Floral leaf length/width ratio
8	Pedicle length (mm)
9	Calyx length (mm)
10	Calyx width (mm)
11	Calyx length/width ratio
12	Calyx teeth length (mm)
13	Inflorescence length (cm)
14	Corolla length (mm)
15	Corolla tube length (mm)
16	Petal length (mm)
17	Corolla tube length/petal length
18	Stamen length (mm)
19	Style length (mm)

Palynology

For Scanning Electron Microscopy examination, pollen grains were transferred directly to a stub with double-sided tape. Then were gold-coated in a JFC-1600 Auto Fine Coater and observed under a Phillips × L20 scanning electron microscope at 15 kV. Features like, shapes the shape of polar and equatorial view and their diameters, aperture and sculpturing shape and dimensions were measured in the electronic micrographs. Pollen terminology follows Erdtman (1952) and Hesse et al. (2009).

Molecular studies

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.

We used 10 primers, $(AGC)_5GG$, $(AGC)_5GT$, $(CA)_7AT$, $(CA)_7GT$, UBC811, UBC810, $(GA)_9T$, $(GA)_9$ A, UBC807 and $(GT)_7CA$ for ISSR examination. The PCR reactions were done in a 25µl volume liquid that have 50 mMKCl, 10 mMTris-HCl buffer at pH 8, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Bioron, Germany), 20 ng genomic extracted DNA, 0.2 µM of a each primer and 3 U of *Taq* DNA polymerase (Bioron, Germany). We used Techne thermocycler (Germany) for PCR reactions and its program was: 5 min initial denaturation at 94 °C, then 40 cycles of 45s at 94 °C; 60 s at 57 °C and 60s at 72°C. At the final stage 7 min extension at 72°C.

The final products were run on 2% agarose gels, then were stained by ethidium bromide. A DNA ladder with100 bp molecular size was used for estimating the size of amplified fragments. We used 3 replications for achievement of reproducible bands.

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′-GTAAGGGT CATTTAGTAGGTCGTTT-3′ and, the reverse primer was 5′-TCCTTACCATTAAGTTGATC-3′. Each 20 µL of 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 Techne thermocycler (Germany) with the following program: 2 min initial denaturation step 94°C, followed by 35 cycles of 5 min at 94°C; 1.30 min at 57°C and 2 min at 72°C. The reaction was completed by 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 (Thermo-Fisher, Waltham, MA, USA).

Data analyses

Morphological and palynological studies

Morphological and palynological data were standardized (Mean = 0, Variance = 1) and used to establish Euclidean distance among pairs of taxa (Podani 2000). For grouping of the plant specimens, The UPGMA (Unweighted paired group using average), Maximum parsimony (MP), PCA (Principal component analysis) were used (Podani 2000). PAST version 2.17 (Hammer et al. 2012) and PAUP* 4.0b10 (Swofford 2002) were used for these analyses. Bootstrapping with 100 times replication was done for maximum parsimony tree.

ISSR analyses

The obtained ISSR bands were coded as binary characters (presence = 1, absence = 0). Nei's genetic distance was determined among the populations studied (Freeland et al. 2011) and used for PCoA ordination (Principal coordinates analysis) (Podani 2000). PAST ver. 2.17 (Hammer et al. 2012) program was used for these analyses.

Two different bioinformatic approaches were used to identify genetic groups in the samples studied: K-Means clustering and Evanno test based on STRUCTURE analysis of ISSR data.

For the Bayesian-based model STRUCTURE analysis (Pritchard et al. 2000) ISSR data were scored as the dominant markers (Falush et al. 2007). An admixture model ancestry with 10^5 times reiteration was used. K-Means clustering was performed as implemented in Geno Dive ver. 2 (2013) (Meirmans and Van Tienderen 2004). AMOVA (Analysis of molecular variance) was performed to investigate genetic difference between two presumed subspecies based on ISSR data.

Cp-DNA sequences analyses

The intron in the gene for ribosomal protein L16 (*rpL16*) was aligned with MUSCLE (Robert 2004) implemented in MEGA 5 and used to study the infra specific relationship by performing using different methods such as UPGMA, and maximum parsimony as performed in MEGA 5 software (Tamura et al. 2011). Networking (Bandelt et al. 1999; Clement et al. 2002; Leigh and Bryant 2015) was done by popart (Population analysis by reticulation trees) (http://popart.otago.ac.nz.).

We constructed the Bayesian tree based on the cp molecular data. For the Bayesian MCMC inferred analyses of the nucleotide sequence data, BEAST v1.6.1 was applied (Drummond et al. 2010a).

Bayesian Evolutionary Analysis Utility version v1.6.1(BEAUti) was used to create initial xml file for BEAST (Drummond et al. 2010b). Nee (2006) have confirmed that a Yule process of speciation , a pure birth' process, can be utilized as a tree prior to all the tree model analyses.

The chain length for the Bayesian MCMC posterior analyses was 10000000. After 100 tree burn-in processing, 10000 trees were used for the analyses. We run the BEAUti xml file (Drummond et al. 2010b) in the BEAST v1.6.1 program (Drummond et al. 2010a). The chain creations of maximum clade credibility (MCC) were repeated five times for all molecular clock models with independent runs to ensure suitable convergence and adequate mixing. The MCC tree was made under the relaxed clock model (HKY

substitution). Because no fossils are available for the species studied, a rate of evolution of the plastid sequence ($u = 1.0 \times 10^{-9}$ s s^{-1} $year^{-1}$) was used. (Zurawski et al. 1984).

Drummond and Rambaut (2007) stated that Tracer v1.5 software can be used for the output of the model parameters to investigate the sampling and convergence data gained from BEAST. We used TreeAnnotator v1.6.1 software to explain the created phylogenetic data by BEAST as a shape of single 'target' tree (Drummond and Rambaut 2007). On the these trees, we have summary statistics of the posterior probabilities (the probability of posterior was 0.5) of each node: the 95% highest posterior density limits of the node lengths, rates as well as the posterior estimates. The FigTree v 1.3.1(Rambaut 2009) program was applied for annotate up the output analyses of BEAST MCC tree.

Furthermore, Wan-Pyo Hong and Jury (2011) have stated that this analysis is similar to the bootstrapping value in Phylogenetic Analysis Using Parsimony analysis (PAUP).

Biogeography

To assess levels of genetic divergence between the two subspecies, quantified pairwise differentiation using Fst and Φst was used. The former looks only at haplotype frequency differences while the latter also incorporates haplotype sequence similarity (Klimova et al. 2017). Fst was calculated by AMOVA as implemented in GenAlex 6.4 (Peakall and Smouse 2006), and Φst values were calculated within popart (http://popart.otago.ac.nz.). Inter-specific genetic distance of Kimura 2 p by MEGA 5 was also determined.

RESULTS AND DISCUSSION

Morphological analysis

MP phylogenetic tree of the morphological data (Figure 1), placed outgroups taxa in three separate clades. They were placed far from the ingroup taxa. Samples of the two presumed subsp. were also placed in separate clades, far from each other. Samples of *Z. capitata* subsp. *orientalis* showed closer to *Z. clinopodioides*, while sample of *Z. capitata* subsp. *capitata* joined them with some distance, and were closer to *Z. persica.*

Plant specimens of each subspecies studied were grouped together, indicating that the subspecies are delimited based on the morphological characters such as floral leaf blade shape, apex and size relative to capitate (Figure 2). PCA analysis of the morphological characters revealed that the two PCA axes comprised about 80% of total variance. In the first axis with about 68% of total variation, characters like, stem length, life form and floral type had the highest positive correlation (>0.70), while calyx length had the highest negative correlation (0.80). Similarly, in the second PCA axis, characters like floral leaf length, corolla tube length and style length had the highest positive correlation (>0.70), while floral leaf had the highest negative correlation (0.90). Therefore, these are morphological characters differentiating the two subsp. within *Z. capitata*.

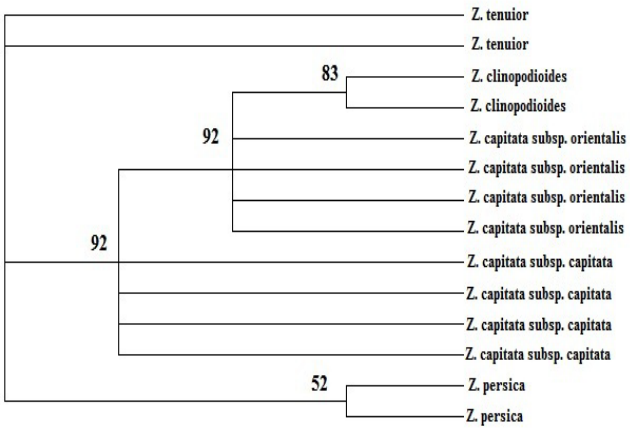

Figure 1. Maximum Parsimony cladogram of the morphological data in *Ziziphora* species.

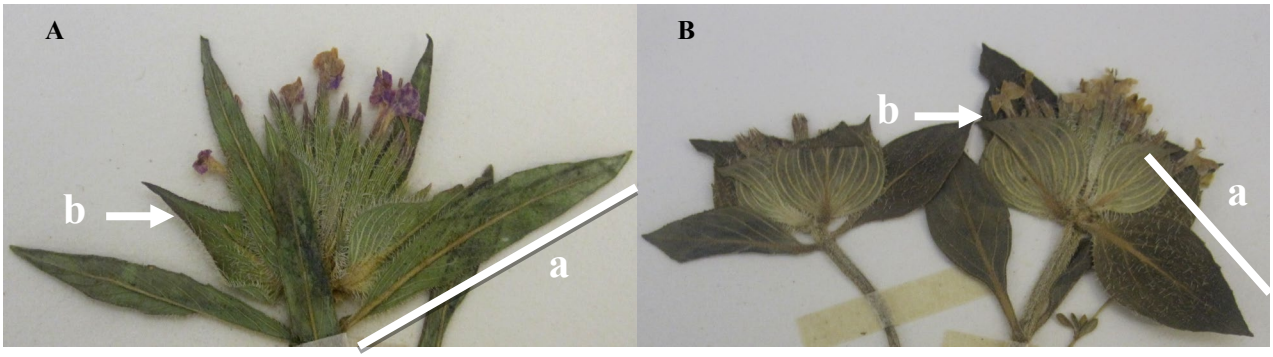

Figure 2. The inflorescence structure in the subspecies studied of *Z. capitata*. A. *Z. capitata* subsp. *capitata*, B. *Z. capitata* subsp. *orientalis*, a. floral leaf shape and b. floral leaf apex

Palynology

Details of pollen characteristics in two subspecies studied are provided in Table 4. and Figure3. Pollen grains of the both *Ziziphora* subsp. are symmetrical, isopolar and hexacolpate monads. The shape of pollen is subprolate, prolate, suboblate and oblate-spheroidal (P/E = 1.21-1.35; Table 4 and Figure 4.) Polar axis length (P) is between 27.68-41.74 µm, and equatorial diameter (E) is between 22.70-31.18 µm.

The pollen grains of *Z. capitata* subsp. *capitata* are bigger than Z. *capitata* subsp. *orientalis.* The colpus length varies from 24.81 to 36.45 µm. It is strongly correlated with length of polar axis. The distances between two colpus vary from 5.78 to 7.17 µm. Apocolpium index diameter ranges from 0.40-0.73 µm. Thickness of muri is between 0.34-0.50 µm.

Exine sculpturing shows two types of surface structures, reticulate-macroreticulate in (*Z. capitata* subsp. *capitata*) and bireticulate-microreticulate in (*Z. capitata* subsp. *orientalis*).

UPGMA of the pollen characters separated the sub-species studied from each other (Figure 5). In this plot, *Z. capitata* subsp. *orientalis* and *Z. capitata* subsp. *capitata* were far from each other, because of these traits such as shape of the pollen (P/E), pore diameter and apocolpium index. So, this result proves our morphology results.

Table 4. The palynological characters studied in *Ziziphora* taxa (all values are in µm).

Characters	***Z. capitata* subsp. *capitata***	***Z. capitata* subsp. *orientalis***	***Z. clinopodioides***	***Z. persica***	***Z. tenuior***
Polar axis length	41.52±0.19	28.04±0.48	25.28±1.32	55.62 ±1.86	27.91±0.62
equatorial axis length	30.81±0.61	23.24±0.82	26.93±1.95	41.26±6.73	32.37±0.27
P/E	1.35	1.21	0.94	1.35	0.86
Pollen shape	Prolate	Subprolate	Oblate-spheroidal	Prolate	Suboblate
Ornamentation	Reticulate	Bireticulate	Bireticulate	Reticulate	Bireticulate
Ornamentation margo	Macroreticulate	Microreticulate	Microreticulate	Macroreticulate	Microreticulate
Distance between colpus	6.46±0.24	6.55±0.57	6.02±1.08	9.76±2.29	7.07±1.68
Colpus length	36.27±0.19	24.89±0.35	24.22±0.31	49.04±3.65	20.65±0.25
AI	0.56±	0.58	0.59±0.84	0.57±1.50	0.0.53±1.01
Thickness of muri	0.46±0.05	0.33±0.02	0.43±0.08	0.40±0.10	0.34±0.07
Pori diameter	0.38±0.08	0.20±0.03	0.40±0.11	0.50±0.21	0.38±0.08

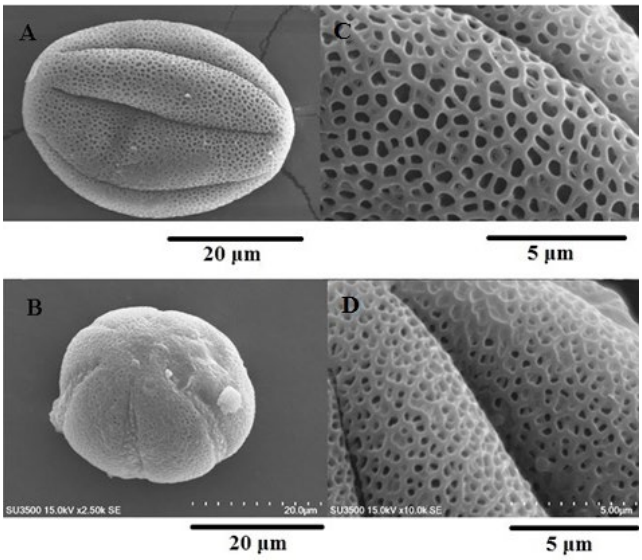

Figure 3. Electronic micrograms of pollen grains in the *Z. capitata* (A) Equatorial view and (C) exine ornamentation of Z. *capitata* subsp. *capitata***.** (B) Polar view and (D) exine ornamentation of *Z. capitata* subsp. *orientalis*.

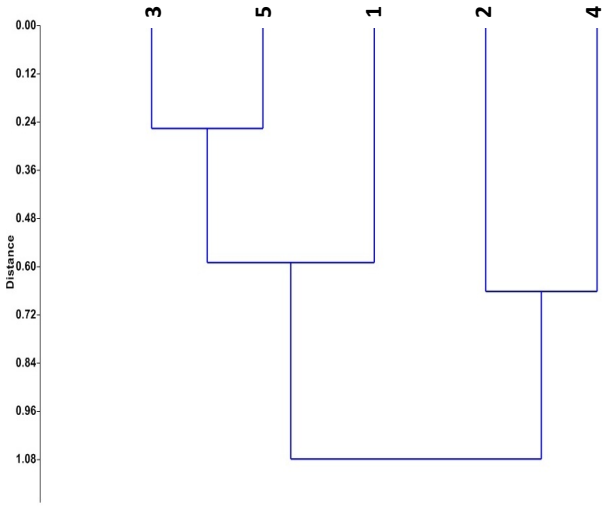

Figure 4. UPGMA of the *Z. capitata* subspecies studied based on the palynological data. Note: 1. *Z. capitata* subsp. *orientalis*, 2. *Z. capitata* subsp. *capitata*, 3. *Z. clinopodioides,* 4. *Z. persica*, 5. *Z. tenuior*

Molecular analyses

ISSR assay

ISSR analysis produced 50 bands. Few ISSR bands were common in both subsp., while most of the bands almost occurred in either of the subsp., and were private bands. These private bands have been probably formed during subsp. divergence, while the common bands are ancestral shared alleles.

The AMOVA test produced significant difference between the subsp. studied in ISSR data. This indicates molecular difference between these taxa. AMOVA revealed that about 21% of total genetic variability occurs due to between sub-species while about 79% of genetic variability is due to within subsp.

K-Means clustering and Evanno test based on the STRUCTURE analysis of ISSR data produced k = 2 as the optimal genetic groups. Moreover, the PCoA plot of the ISSR data (Figure 5) separated the two presumed subsp., in two groups.

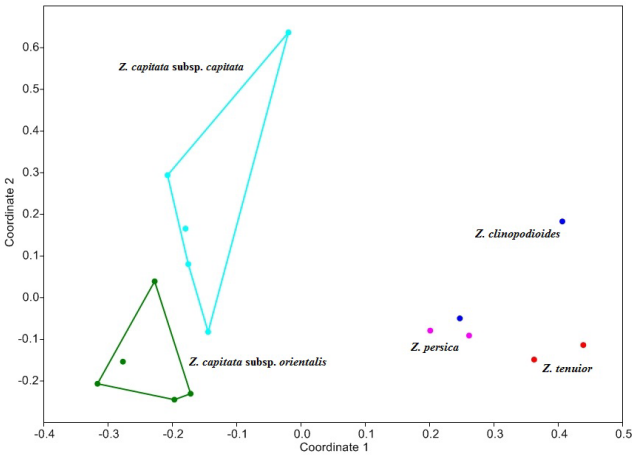

Figure 5. PCoA plot of the *Z. capitata* subspecies studied based on the ISSR data

CP-DNA analyses

Cp-DNA analysis produced following statistics: Nucleotide diversity: pi = 0.457082, the number of segregating sites =170, the number of parsimony-informative sites = 34, Tajima's D statistic: D = 1.55726 (p (D >= 1.55726). Moreover, AMOVA based on cp-DNA sequences produced Φst st = 1.0 (p = 0.01) indicating significant molecular difference between the subsp. studied.

Different phylogenetic analyses based on the MP, Bayesian approach and networking (Figs 6-8) revealed that the two subsp. are well differentiated on the genetic grounds. In both MP and Bayesian trees, samples of the two subsp., were placed far from each other. *Z. capitata* subsp. *capitata* was placed close to *Z. tenuior*, and far from *Z. capitata* subsp. *orientalis*. In both analyses, *Z. capitata* subsp. *orientalis* showed closer affinity with *Z. persica*. Genetic distance of the two subsp. was estimated to be 1.20 by Kimura 2p distance.

In the present study, morphological, palynological and molecular ISSR AND cp-DNA sequences were used for investigation of infraspecific variations in *Z. capitata*. Palynology is one of the important tools in taxonomy of Lamiaceae family, which provide valuable data on classifications of genera and species within this family. Erdtman (1945) investigated pollen of Lamiaceae and divided this family into two subfamilies. Furthermore, recent studies show that palynological characters are very useful in taxonomy of the genus *Ziziphora* (Keshavarzi et al. 2008).

The observed pollen grains are hexacolpate, prolate (*Z. capitata* subsp. *capitata*) to subprolate (*Z. capitata* subsp. *orientalis*). Obtained results confirmed Selvi et al. (2013) findings. They stated that the pollen grains of *Z. capitata* are hexacolpate, suboblate to prolate spheroidal in shape. Moreover, pollen exine ornamentation was reticulate in *Z. capitata* subsp. *capitata* and bireticulate in *Z. capitata* subsp. *orientalis*. While, Selvi et al. (2013) have suggested that and ornamentation in this species is perforate.

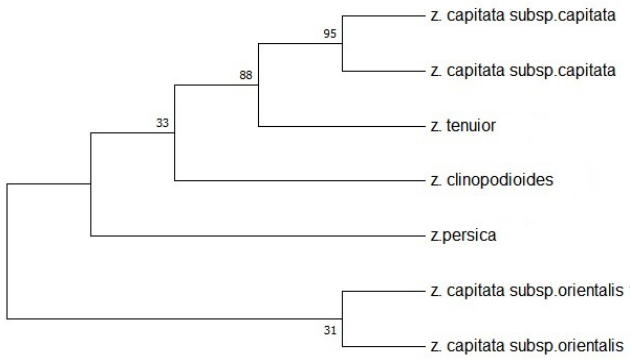

Figure 6. Maximum parsimony tree of the *Ziziphora* species studied based on cp-DNA sequences

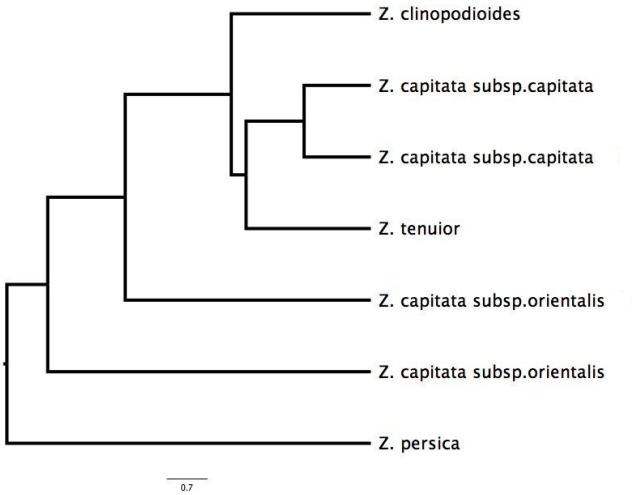

Figure 7. Bayesian tree of the *Ziziphora* species studied based on cp-DNA sequences

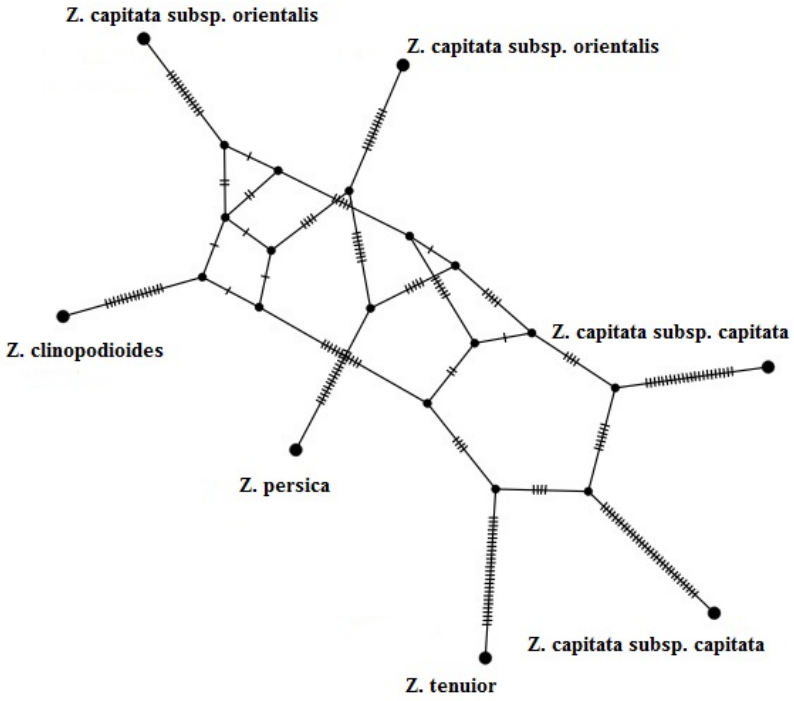

Figure 8. Integer NJ net of the *Ziziphora capitata* subspecies studied based on the cp-DNA sequences

Palynological characteristics highly differed between the subspecies studied, and they placed separately in the UPGMA tree of palynological data. Previous studies (e.g. Talebi et al. 2012, 2014, 2016) proved that palynological study provides valuable data for identifications of very similar taxa and are useful in the alternations of taxonomical ranks.

Morphological characters of plants can be compared, measured, counted and described to assess the differences or similarities in plant taxa and these characters are used for plant identification, classification and description, thus, solving taxonomic problems (Woodger 1967; Raven et al. 2005). The taxa studied were separated in the Maximum Parsimony cladogram of the morphological. It confirmed high morphological difference among taxa. PCA analyses showed some morphological traits widely varied among the species/ subspecies studied. Sattler (1994) have stated that the morphology is comparative science and botanists try to study morphological structures in several plant specimens of the same or different taxa in order to reach a suitable descriptions and keys about similarities or differences.

ISSR molecular technique has been successfully used for investigation of infraspecific variations in different genera in Lamiaceae such as *Stachys* (Sheidai et al. 2016), *Lallemantia* (Koohdar et al. 2016) or other Families such as Linaceae (Sheidai et al. 2014). Therefore, we decided to use the molecular approach for investigation of infraspecific variations between *Z. capitata* subspecies. Our results confirmed separation of these subspecies. In the plot of ISSR data samples of each subspecies placed separately, moreover, they were far from other *Ziziphora* species. AMOVA test exhibited significant genetic variation between the subspecies and outgroup species, it shows high genetic variations among the *Z. capitata* subspecies and other species of the genus.

Plant molecular studies enormously advanced in the recent years and molecular phylogenetic investigation has dramatically reshaped our views of organismal relationships and evolution at all taxonomic levels of the hierarchy of life, from the species level (and below) to kingdom (and above). However, it is evident that reliance on a single data set may result in insufficient resolution or an erroneous picture of phylogenetic relationships. As a result, it is now common practice to use multiple data sets (preferably both molecular and none molecular) for phylogenetic inference (Soltis and Soltis 2000).

Bayesian and maximum parsimony phylogenetic trees differentiated the presumed sub-species within *Z. capitata*. They also differed significantly in both multilocus data (ISSRs) and cp-DNA sequences and showed greater among taxa genetic distance compared to within taxa distance. Moreover, K-Means clustering and Bayesian analysis of genetic groups by Evanno test (optimal k) supported the presence of two distinct genetic groups within *Z. capitata*. This approach objectively defines a threshold separating intraspecific population substructure from interspecific divergence which is the general aim of species delimitation studies (Sites and Marshall 2004). Therefore, we believe that the present study provides enough evidence for splitting *Z. capitata* into two separate species based on traditional taxonomy as well as combination of molecular and micro-morphological data.

Conclusion

The present study revealed the great difference between two presumed subsp., within *Z. capitata*. These taxa differ in taxonomically important morphological, palynological and molecular characteristics. Statistical and bioinformatics tests revealed the significant genomic difference between them and phylogenetic analyses showed that they have closer affinity to some of the other *Ziziphora* species (outgroups used), than to each other. Therefore, the multiple data analysis indicates active speciation event within *Z. capitata* and encourage us to consider these two presumed subspecies as two separate and distinct species.

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