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Species relationship and genetic diversity in some Iranian *Lamium* L. species using ISSR markers

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Abstract. *Azimishad F, Sheidai M, Talebi SM, Noormohammadi Z. 2019. Species relationship and genetic diversity in some Iranian* Lamium *L. species using ISSR markers. Biodiversitas 20: 1963-1972.* Lamium is a widespread and taxonomically complex genus of Lamiaceae which comprises of 16-38 species. This genus is represented in Iran by nine species. In the present study, we used morphological and molecular (ISSR, Cp DNA, and nrITS) data to evaluate species relationships, genetic diversity and population genetic structure of the genus. 27 morphological characteristics, including 13 qualitative and 14 quantitative, and ten ISSR markers were used for morphological and genetical evaluation of 73 accessions from eight taxa. In general, species relationships obtained from morphological and molecular data were largely congruent. In the morphological study, characteristics like the life form, leaf shape, absence/existence of bracts and shape of corolla, were distinctive traits and we did not encounter intermediate forms. Our findings indicated a very high efficiency of the ISSR markers in the identification and delimitation of *Lamium* species. These results confirmed the placement of *L. galeobdolon* in the genus *Lamium* and segregation of *L. purpureum* and *L. garganicum* in section *Lamium*. AMOVA analysis revealed that the species of this genus are genetically differentiated. Nm analysis showed very low value of gene flow among the studied species and mantel test indicated isolation by distance occurred among them.

Keywords: Genetic diversity, ISSR, *Lamium*, morphology, species delimitation

INTRODUCTION

Lamium L., the type genus of family Lamiaceae, which comprises of 16-38 species, depending on the circumscription of the genus (Briquet 1895-1897; Mennema 1989; Harley et al. 2004; Bendiksby et al. 2011a). The genus includes both annual and perennial herbaceous plants that are widely distributed in the temperate and subtropical regions of Eurasia and Northern Africa, where its center of diversity lies in the Irano-Turanian and Mediterranean regions (Mennema 1989). Deadnettle - the common name of the genus *Lamium*- refers to the similarity of the vegetative parts of *Lamium album* L. to distantly related stinging *Urtica dioica* L. (nettles), however, the members of the genus do not have such stinging hairs and this may suggest a harmless mimicry (Brown et al. 1991).The best diagnostic characters of *Lamium* species are the short and toothed lateral lobes of the lower lip of the corolla and a broad and emarginated middle lobe (Bendiksby et al. 2011a).

Lamium species possess significant pharmacological and biological activities including antioxidant, anti-inflammatory, astringent, antispasmodic, antiseptic and uterotonic properties, which are useful for problems as menorrhagia, paralysis, hypertension, chronic bronchitis, prostrate and scrofula (Bremness 1995; Baytop 1999). Aerial parts of these plants are used as medicine by locals in Iran (Akbarzadeh et al. 2010; Yazdanpanah et al. 2013). Also, some *Lamium* species are grown as ornamental plants in gardens and parks (Rudy 2004).

Nine *Lamium* species occur in Iran (Jamzad 2012): *Lamium galeobdolon* (L.) L.; *L. amplexicaule* var. *amplexicaule* L.; *L. amplexicaule* var. *aleppicum* (Boiss. & Hausskn. ex Boiss.) Bornm.; *L. amplexicaule* var. *bonmulleri* Mennema.; *L. macrodon* Boiss. & A. Huet.; *L. album* subsp. *album* L., *L. album* subsp. *crinitum* (Montbr. & Auch. Ex Benth.) Mennema.; *L. bakhtiaricum* Jamzad.; *L. tomentosum* Willd., *L. persepolitanum* (Boiss.) Jamzad.; *L. garganicum* L. and *L. purpureum* L., out of which *L. bakhtiaricum* and *L. persepolitanum* are endemic for Iran. In addition, *L. macrodon* considered as the endangered species (Jalili and Jamzad 1999). Systematic position of *L. galeobdolon* is controversial. Some botanists (Mossberg et al. 1992; Ryding 2006; Stace 2010) placed the species in genus *Lamiastrum* Heist. Ex Fabr., whereas others (Harley et al. 2004; Govaerts et al. 2010) included *L. galeobdolon* in *Lamium*. Molecular phylogenetic study investigated by Bendiksby et al. (2011a) showed that *L. galeobdolon* should be included in *Lamium.*

Mennema (1989) in his taxonomic revision of *Lamium*, recognized three subgenera: (i) subgenus *Orvala* (L.) Briq., (ii) subgenus *Galeobdolon* (Adans.) Asch. and (iii) subgenus *Lamium* L. Within subgenus *Lamium*, he determined the following three sections: sect. *amplexicaule* Mennema, sect. *Lamium* and sect. *Lamiotypus* Dumort. However, the latest molecular phylogenetic study (Bendiksby et al. 2011a) suggests that Mennema's (1989) infrageneric classification should be abandoned.

Genetic diversity is an important element in the survival of population, because it has a positive influence on the adaptation of species in the presence of fluctuating biological and abiotic factors (Vrijenhoek 1994). ISSR (Inter-simple sequence repeats) molecular markers are stable, highly reproducible, easy to work and are known to be useful in various biological investigations such as, species delimitation, genetic diversity analysis, population structure, breeding programs and conservation management (Shahi-Gharahlar et al. 2011; Sheidai et al. 2014; Koohdar et al. 2015; Minaeifar et al. 2016; Nikzat-Siahkolaee et al. 2017; Tabaripour et al. 2018).There are many studies which have been mainly focused on the taxonomy, phylogeny, nutlet micromorphology, cytology, anatomy, trichome and pollen morphology of *Lamium* species (Mill 1982; Mennema 1989; Abu-Asab and Cantino 1994; Baran and Özdemir 2009, 2011; Bendiksby et al. 2011a, b; Celep et al. 2011, 2014; Krawczyk et al. 2013, 2014; Atalaya et al. 2016 a, b). However, genetic diversity of *Lamium* species have been reported in only a few studies (Wąsowicz et al. 2011; Stojanova et al. 2013; Azimishad et al. 2018), also genetic structure, ecological adaptation, and intra- and inter-specific differentiation along with morphometric studies on *Lamium* of Iran have not been investigated yet.

Therefore, we performed morphological and molecular study of 73 collected accessions in the genus *Lamium* to following objectives: (i) to evaluate the species relationships in order to compare our result with the recent phylogenetic study carried out in the genus *Lamium* to provide new information in support of the current taxonomic classifications. (ii) to analyses genetic diversity and population structure to answer the following questions: (i) Is there infra and interspecific genetic diversity among the studied species? (ii) Is genetic distance among these species correlated with their geographical distance? (iii) What is the genetic structure of populations and taxa?

MATERIALS AND METHODS

Plant material

In the current study, 73 plant specimens were studied from 10 geographical populations located in six provinces of Iran (Table 1, Figure 1). The sampling locations and identification of *Lamium* species were based on the descriptions provided by Flora Iranica (Rechinger 1982) and Flora of Iran (Jamzad 2012).

Morphological studies

For morphological studies, 5-10 samples from each taxon were used. In total 27 morphological characters including 13 qualitative and 14 quantitative were studied (Table 2).

DNA extraction and PCR amplification

Fresh leaves were used randomly from 2–10 plants in each of the examined populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA (Sheidai et al. 2013). Ten ISSR primers: $(AGC)_5GT$, $(CA)_7GT$, $(AGC)_5GG$, UBC810, $(CA)_7AT$, $(GA)_9T$, $(GT)_7CA$, $(CA)_7AC$, $(GA)_9A$ and $(GA)_9C$ commercialized by UBC (the University of British Columbia) were used for polymorphism detection on the accessions. PCR reactions were carried out in a 25 µl volume containing 10 mM Tris-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 a single primer; 20 ng genomic DNA and 3 U of Taq DNA polymerase (Bioron, Germany). The amplification reactions were performed in Techne thermocycler (Germany) with the following program: 5 Min initial denaturation step 94˚C, followed by 40 cycles of denaturing: 30 s at 94 ˚C, 1 Min at 53-58˚C, and 1Min at 72 ˚C. The reaction was completed by the final extension step of 7 Min at 72˚C.

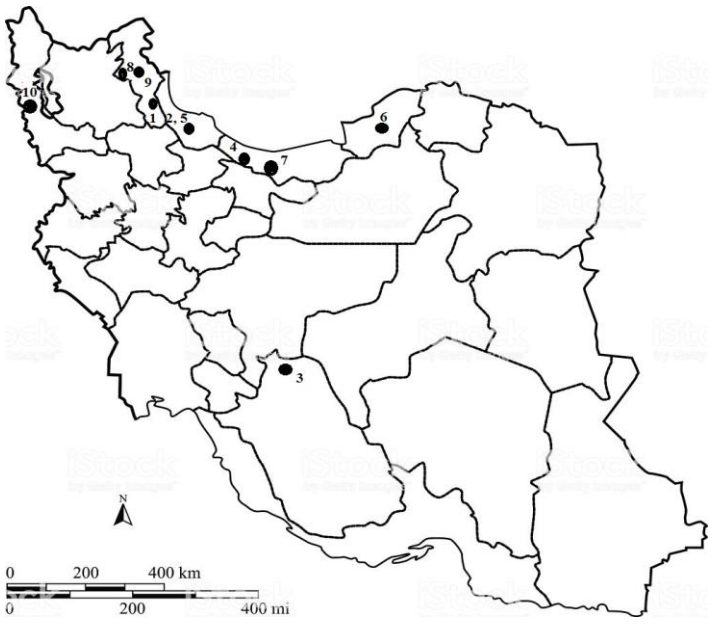

Figure 1. Distribution map of the studied *Lamium* species (Numbers are taxa names according to Table 1).

Table 1. Localities addresses of the studied *Lamium* taxa and their populations in Iran

R	Taxa	Locality	Alt (m)	Latitude	Longitude	Voucher No
1	*L. amplexicaule* var. *amplexicaule*	Ardabil Province, Khalkhal-Asalem Road	1500	37° 57′ 36″	48° 61′ 03″	HSBU102
2	*L. amplexicaule* var. *amplexicaule*	Guilan Province, Damash	1700	36° 75′ 54″	49° 81′ 07″	HSBU101
3	*L. amplexicaule* var. *aleppicum*	Fars Province, Shahr miyan	2700	30° 84′ 40″	52° 06′ 76″	HSBU113
4	*L. galeobdelon*	Mazandaran Province, Chalus, Visar	1400	36°65 ′011′	51°31′051 ′	HSBU2019107
5	*L. purpureum*	Guilan Province, Damash	1700	36° 75′ 54″	49° 81′ 07″	HSBU2019108
6	*L. album* subsp. *crinitum*	Golestan Province, Golestan Forest	700	37°47′ 50 ″	47°23′36.2′	HSBU2019109
7	*L. album* subsp.*crinitum*	Mazandaran Province, Noshahr, Kheyrud kenar Forest	400	36° 38' 05"	51° 29' 05 ′	HSBU2019110
8	*L. album* subsp. *album*	Ardabil Province, Meshkin shahr, Hatam Forest	2700	38°18′77.1′	56°41′ 60 ″	HSBU2019111
9	*L. tomentosum*	Ardabil Province, Meshkin shahr, Sabalan MT, Shahbil, Qotursooi village	2800	38°17′ 00 ″	47° 50 ′ 00 ″	HSBU2019112
10	*L. garganicum* subsp. *striatum*	West Azerbaijan Province, Khoy, Pasak, Badalan Village, waterfall	1700-2000	34° 45 ′ 50 ″	45° 00 ′ 00 ″	60835/3 IRAN

Table 2. Morphological characteristics in the studied species

Morphological characteristics
Life form
Plant height(cm)
Absence/existence petiole in inflorescence leaf
Length of petiole (mm)
Length of basal leaf(mm)
Width of basal leaf (mm)
Ratio of basal leaf length/basal leaf width (mm)
Length of floral leaf (mm)
Width of floral leaf (mm)
Ratio of floral leaf length /floral leaf width
Absence/presence of bracts
Size of bract(mm)
Trichome of bract
Shape of basal leaf
Shape of floral leaf
Length of calyx (mm)
Length of calyx dent(mm)
Length of calyx tube(mm)
Ratio of Calyx teeth length/ Calyx tube length(mm)
Shape of calyx dent
Corolla color
Shape of corolla
Shape of corolla tube
Length of corolla(mm)
Absence/presence fluff on the anther
Corolla with/without the fluff ring
Flower cycle

The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analysis

Morphological analysis

Morphological characteristics were standardized (Mean = 0, variance = 1) and used to estimate Euclidean distance for clustering and ordination analyses (Podani 2000). For clustering of the plant specimens, UPGMA (unweighted paired group using average) method was used. Different ordination methods were applied on standardized data like, PCA (Principal components analysis), and MDS (Multi-dimensional scaling) (Podani 2000). PAST version 2.17 (Hamer et al. 2012) was used for multivariate statistical analyses of morphological data.

ISSR analysis

The polymorphic bands scored as present (1) or absent (0). Nei's genetic distance was determined among the studied taxa and used for clustering. The species grouping was done by UPGMA (unweighted pair-group method) clustering method (Podani 2000), using PAST (Paleontological statistics software package) version 2.17 (Hamer et al. 2012). Moreover, genetic differentiation of the species was determined by AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse 2006). Genetic polymorphism of the studied species was determined by using genetic diversity parameters: Nei's gene diversity (H), Shannon information index (I), the number of effective alleles, and percentage of polymorphism (Weising et al. 2005; Freeland et al. 2011). Gene flow was determined by calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm = 0.5(1 - Gst)/Gst. The Mantel test was performed to check correlation between geographical distance and the genetic distance of the studied species (Podani 2000). PAST ver. 2.17 (Hamer et al. 2012) programs were used.

Cp- DNA and ITS sequences analyses

All sequences including plastid (*psbA-trnH* spacer) and nrITS sequences were obtained from GenBank. We used *psbA-trnH* chloroplast DNA spacer, because it was most effective single-locus barcode in the identification of *Lamium* species (Krawczyk et al. 2013).

Table 3. List of specimens used in the present study including GenBank accession numbers.

Taxon	GenBank accession number	
	nrITS	*psbA-trnH* spacer
L. . amplexicaule var. *amplexicaule*	KP338094	JF780120
	AY443449	JF780121
	JX073975	JF780122
	JX073976	JF780123
	JX073976	
L. . amplexicaule var. *aleppicum*		JF780117
		JF780118
		JF780119
L. galeobdelon	KC350628	JF780148
	KC350627	JF780147
	JX073962	JF780146
L. purpureum	JX073973	JF780180
	JX073971	JF780181
	JX073972	JF780183
		JF780182
L. album subsp .*crinitum*		JF780116
L. album subsp. *album*	KF055050	JF780110
	JX073964	JF780112
	KF529537	JF780111
	JX893229	
L. tomentosum	KC350641	JF780184
	KC350639	JF780186
	KC350640	JF780185
L. garganicum	KC350632	JF780152
	JX073981	JF780153
Eriophyton wallichii	KF769023	JN044481
	JF976304	JF780109
Roylea cinerea	KF769027	JF780106
	KF769028	

The outgroup was selected based on the results from previous phylogenetic studies of subfamily Lamioideae (Scheen et al. 2010; Bendiksby et al. 2013). Their accession numbers are provided in Table 3.

All sequences were aligned and cured. These were then used to construct Maximum Parsimony (MP) phylogenetic tree. Sequence alignment and maximum parsimony method were done by MEGA 5 software (Tamura et al. 2011). Bootstrapping was performed with 1000 replications.

RESULTS AND DISCUSSION

Morphometry

In order to determine the most variable characters among the taxa examined, PCA analysis was performed. It revealed that the first three factors comprised over 82% of the total variation. In the first PCA axis with 56% of the total variation, characteristics such as life form, absence/existence petiole in inflorescence leaf, length and width of leave and length of calyx tube (>0.7) had the highest value of correlation. Shape of leave, absence/existence of bracts and corolla with/without the fluff ring, were characteristics influencing PCA axis 2 and 3. Different clustering and ordination methods produced similar results therefore, UPGMA clustering and MDS plot of morphological characteristics were presented here (Figures 2, 3). In UPGMA tree (Figure 2), plant samples of each species, were grouped together and formed separate cluster. Among the studied specimens, we did not encounter intermediate forms. These results showed that morphological characteristics studied can differentiate the *Lamium* species in two different major clusters or groups. The first major cluster that was supported by significant bootstrapping values of higher than 50%, and was divided into two main sub-clusters so that the plants of *L. amplexicaule* var. *amplexicaule* (P1- P 2) and *L.amplexicaule* var. *aleppicum* (P3) comprised the first sub-cluster (sect. *amplexicaule*) due to morphological similarity, while the plants of *L. purpureum* formed the second sub-cluster (sect. *Lamium*). Similarly, the second major cluster included two sub-clusters: the first sub-cluster contained *L. garganicum* (P10, sect. *Lamium*) and *L. tomentosum* (P9, sect. *Lamiotypus*) species, while the plants of *L. album* (P6- P8, sect. *Lamiotypus*) and *L. galeobdelon* (P4) were grouped in the second sub-cluster.

The MDS plot of the morphological characteristics (Figure 3) separated these species into distinct groups with no inter-mixing. This was similar to UPGMA tree that presented before.

Genetic diversity

Ten ISSR primers produced 70 bands across the 73 accessions, of which 27 were private. *L. amplexicaule* var. *amplexicaule* (P1) had the highest private bands (15), while populations 6-10 did not produce any private bands. Over the 10 primers, fragment size ranged from 100 to 2000 bp.

Genetic diversity parameters determined in the studied species (Table 4) revealed that *L. amplexicaule* var. *aleppicum* (P3) had the highest level of genetic polymorphism (44.29%), while the lowest level of genetic polymorphism (4.29%) occurred in *L. album* subsp. *crinitum* (P6). *L. amplexicaule* var. *aleppicum* also had the highest values for effective number of alleles (Ne = 1.183) and Shannon information index (I = 0.18).

AMOVA test showed significant genetic difference (P = 0.001) among the studied species. It revealed that 51% of the total variation was among species and the rest was within species.

The studied taxa and their populations place separately in UPGMA tree base on the ISSR data (Figure 4). This tree was very similar with morphological tree (except *L. galeobdelon* place) and separated plants of each species in a single cluster with no inter-mixing. This is in agreement with AMOVA and genetic diversity parameters presented before. The Nm analysis by Popgene software also produced mean Nm= 0.27, that is considered very low value of gene flow among the studied species. Mantel test with 5000 permutations showed a significant correlation (r = 0.18, p = 0.002) between genetic distance and geographical distance, so isolation by distance (IBD) occurred among the *Lamium* species studied. Nei's genetic identity and the genetic distance were determined among the studied species (Table 5). The results showed that the

highest degree of genetic similarity (0.95) occurred between *L. purpureum* and *L. galeobdelon* . The lowest degree of genetic similarity occurred between *L. album* subsp. *crinitum* and *L. amplexicaule* var. *amplexicaule* (0.71).

Cp- DNA and ITS

Lengths of the aligned regions were: 528bp for *psbA-trnH* spacer and 710bp for the nrITS. Numbers of parsimony informative characters were 120 and 228 for the Cp- DNA, and nrITS, respectively.

Maximum parsimony (MP) tree of the studied *Lamium* species based on the cp-DNA sequences contained two major clusters (Figure 5). All species belonging to sect. *Lamiotypus* and *L. galeobdolon*, were placed in the first major cluster. The second major cluster contained two sub-clusters. Accessions of *L. purpureum* comprised the first sub-cluster, while members of sect. *Amplexicaule* and sect. *Lamium* were placed intermixed together and formed the second sub-cluster.

In the MP tree of the nrITS (Figure 6), all accessions of *L. galeobdolon* were placed with great distance from the other species and formed the first major cluster. In the second major cluster, all species of each section were grouped together and formed separate clade.

Tree topologies

In general, species relationships obtained from the morphological and molecular data were largely congruent (Figures 2, 4-6); however, some incongruent patterns could be identified between the four datasets. For example, in the morphological and ISSR trees (Figures 2, 4), *L. garganicum* showed a close relationship with *L. album* and *L. tomentosum*, in the chloroplast and nuclear trees (Figures 5, 6), however, this species appeared along with *L. amplexicaule* var. *aleppicum* and *L. purpureum*, respectively. Moreover, position of *L. galeobdolon* within *Lamium* species varied between all trees. In the morphological and cpDNA trees (Figures 2, 5), *L. galeobdolon* appeared along with *L. album* and *L. tomentosum* in the main clade, whereas in the nuclear tree (Figure 6), a clade comprising all accessions of *L. galeobdolon* obtained a position as phylogenetic sister to all remaining *Lamium* species. Dendrogram based on ISSR data (Figure 4), showed a close genetic affinity between *L. galeobdolon* and *L. purpureum.* Close relationship between *L. amplexicaule* var. *amplexicaule*. and *L. amplexicaule* var. *aleppicum* and also between *L. album* and *L. tomentosum* could be identified between all datasets.

Table 4. Genetic diversity parameters in the studied populations

R	Taxa	N	Na	Ne	I	He	UHe	P %
1	*L. amplexicaule* var. *amplexicaule*	10	0.83	1.170	0.172	0.110	0.126	35.71%
2	*L. amplexicaule* var. *amplexicaule*	10	0.814	1.180	0.177	0.113	0.124	38.57%
3	*L. amplexicaule* var. *aleppicum*	11	0.886	1.183	0.184	0.116	0.122	44.29%
4	*L. galeobdelon*	8	0.686	1.157	0.148	0.096	0.103	31.43%
5	*L. purpureum*	8	0.643	1.173	0.154	0.102	0.109	30.00%
6	*L. album* subsp. *crinitum*	5	0.243	1.033	0.026	0.018	0.022	4.29%
7	*L. album* subsp. *crinitum*	8	0.400	1.087	0.076	0.051	0.057	14.29%
8	*L. album* subsp. *album*	6	0.286	1.046	0.040	0.027	0.032	7.14%
9	*L. tomentosum*	5	0.400	1.112	0.090	0.062	0.069	15.71%
10	*L. garganicum*	2	0.271	1.032	0.030	0.020	0.024	5.71%

Note: Na = No. of Different Alleles, Ne = No. of Effective Alleles, I = Shannon's Information Index, He = Expected Heterozygosity, P % = Percentage of Polymorphic Loci

Table 5. Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Pop ID	1	2	3	4	5	6	7	8	9	10
1	****	0.8368	0.8583	0.8316	0.8494	0.7198	0.7520	0.7546	0.7848	0.7559
2	0.1781	****	0.9105	0.8758	0.8892	0.7961	0.8139	0.8098	0.8643	0.8476
3	0.1528	0.0937	****	0.9195	0.9356	0.8539	0.8709	0.8522	0.8662	0.8691
4	0.1843	0.1327	0.0839	****	0.9576	0.8116	0.8173	0.8293	0.8259	0.8794
5	0.1632	0.1175	0.0666	0.0434	****	0.8044	0.8411	0.8258	0.8397	0.8644
6	0.3288	0.2280	0.1579	0.2087	0.2176	****	0.8993	0.8553	0.8642	0.7733
7	0.2851	0.2059	0.1383	0.2018	0.1731	0.1061	****	0.8703	0.8741	0.8152
8	0.2816	0.2110	0.1599	0.1872	0.1915	0.1563	0.1389	****	0.9451	0.8329
9	0.2424	0.1458	0.1437	0.1912	0.1747	0.1459	0.1346	0.0565	****	0.8479
10	0.2799	0.1653	0.1403	0.1285	0.1457	0.2571	0.2044	0.1828	0.1649	****

Note: Population codes are according to Table 1

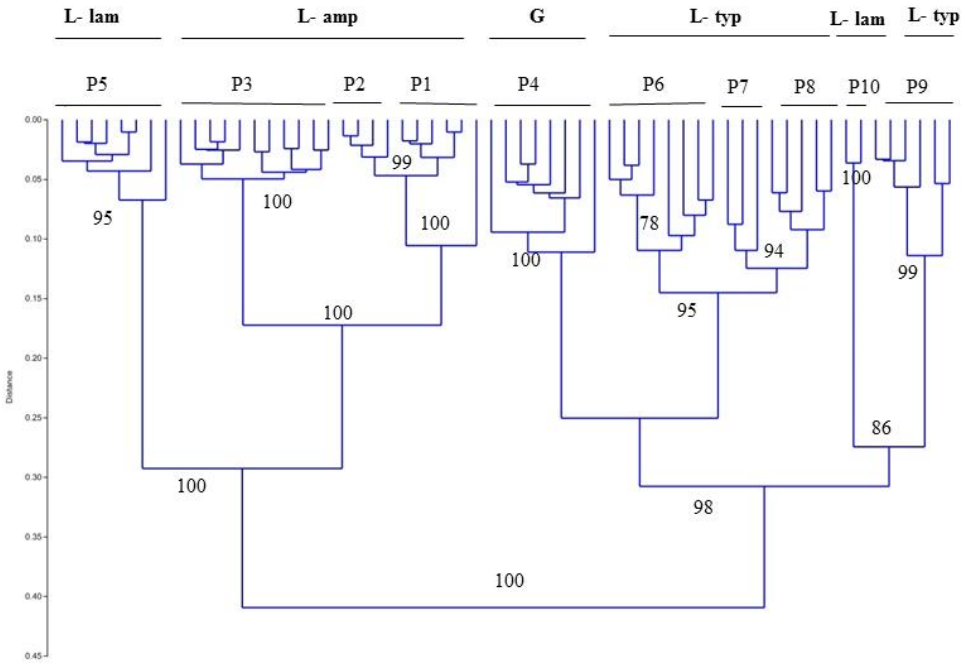

Figure 2. UPGMA clustering of the morphological characters revealing species delimitation in *Lamium* species. Bootstrap value from1000 replicates are indicated below branches, P1-P2 = *L. amplexicaule* var. *amplexicaule*; P3 = *L.amplexicaule* var. *aleppicum*; P4 = *L. galeobdelon*; P5 = *L. purpureum*; P6-P7 = *L. album* subsp. *crinitum*; P8 = *L. album* subsp. *album*; P9 = *L. tomentosum* and P10 = *L. garganicum*. Abbreviations to the above refer to Mennema's (1989) infrageneric classification: G = subg. *Galeobdolon*; L = subg. *Lamium*; amp = sect. *Amplexicaule*; lam = sect. *Lamium*; typ = sect. *Lamiotypus*

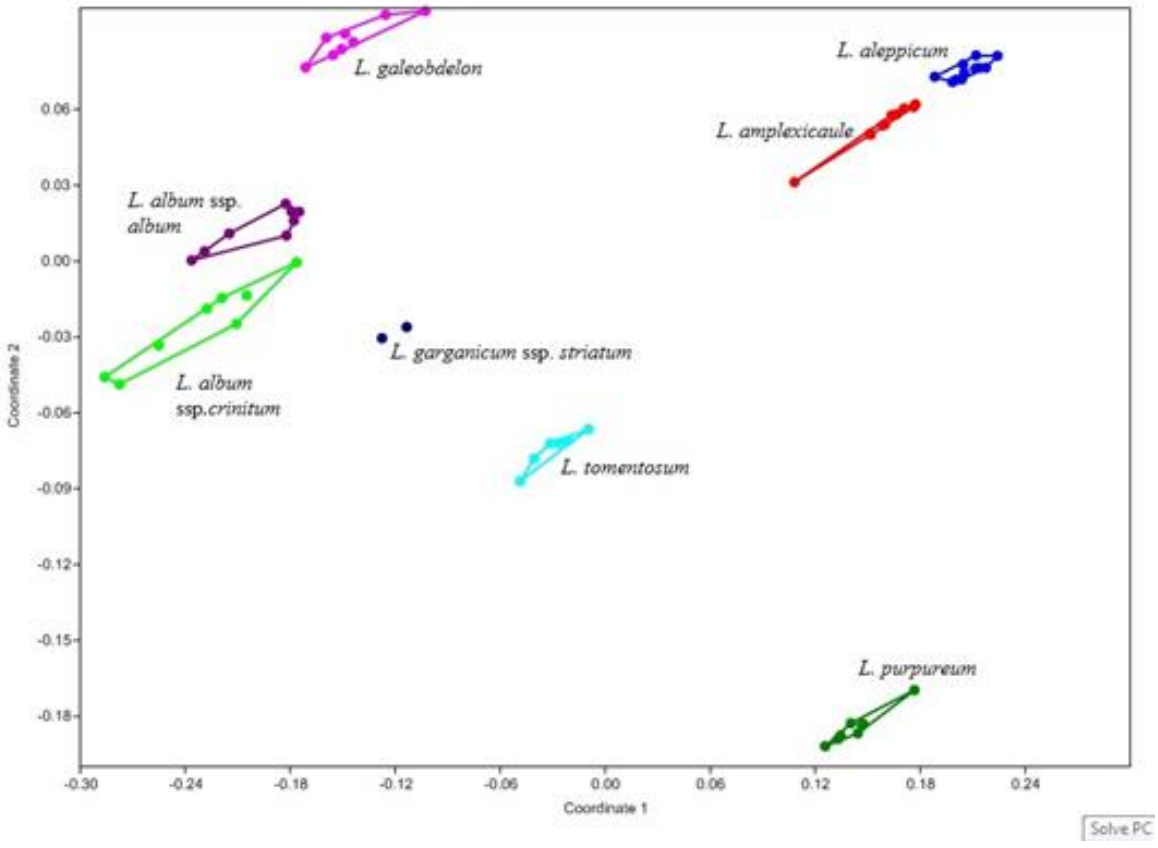

Figure 3. The MDS plot of *Lamium* taxa based on the morphological data

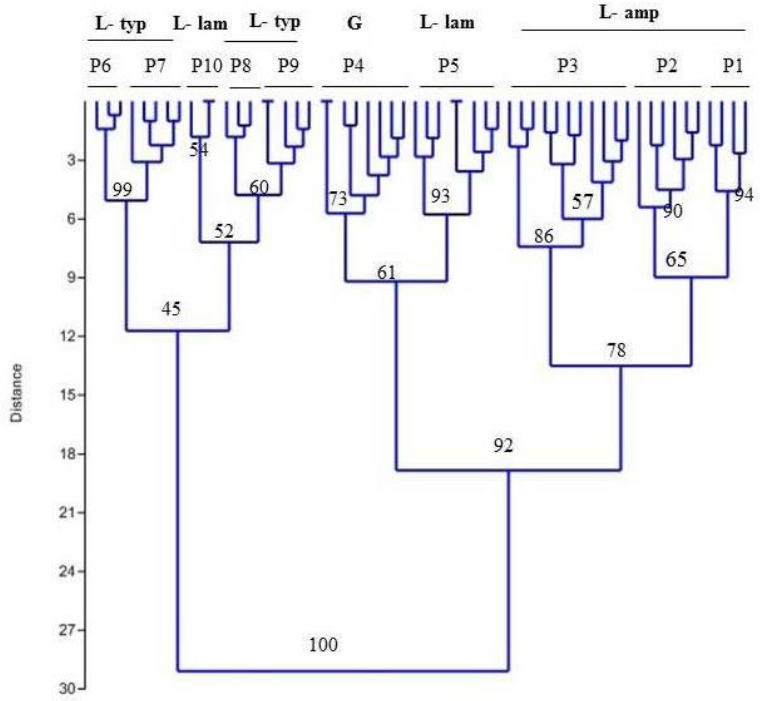

Figure 4. UPGMA dendrogram of ISSR data, showing species delimitation in *Lamium* species. Note: Population codes are according to Table 1, Abbreviations and branch support as in Figure 2

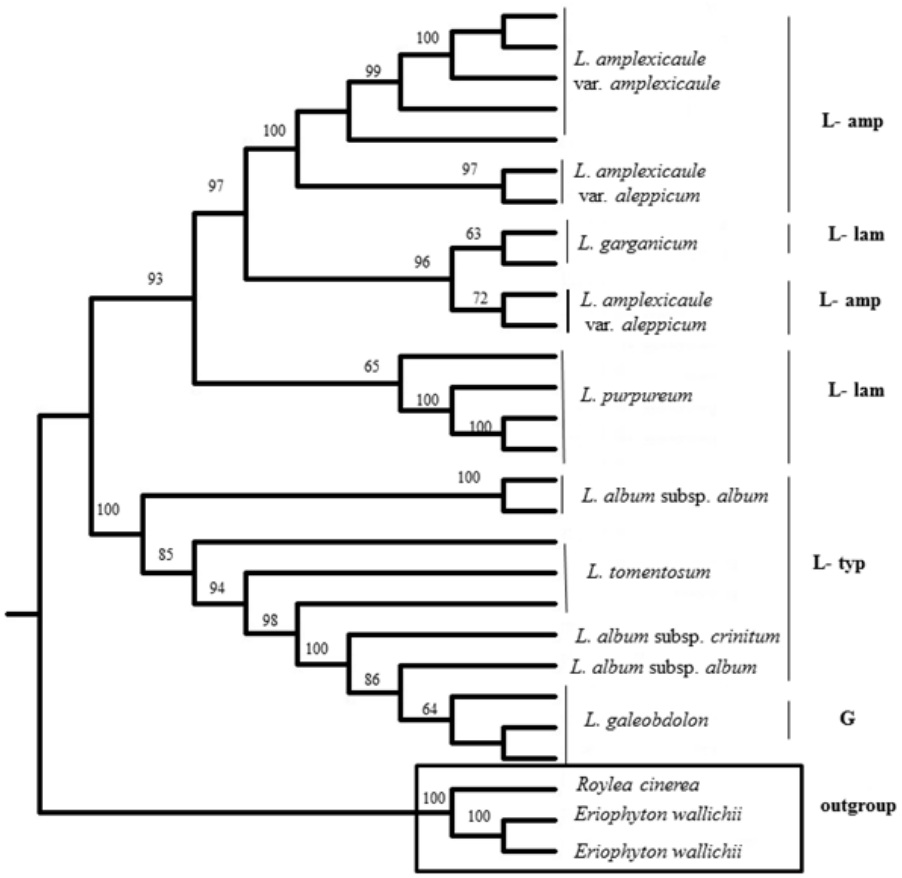

Figure 5. Maximum parsimony tree of the *Lamium* species studied based on cp-DNA sequences. Note: Abbreviations and branch support as in Figure 2

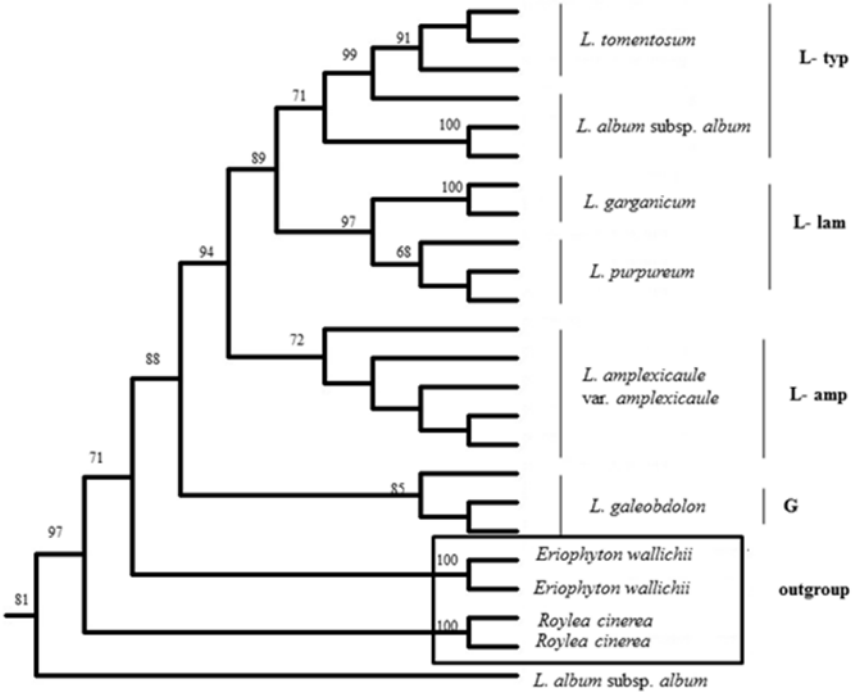

Figure 6. Maximum parsimony tree of the studied *Lamium* species based on ITS sequences. Note: Abbreviations and branch support as in Figure 2

Species identification and relationships

In the present study we used morphological and molecular (ISSR, cpDNA, and nrITS) data to evaluate species relationship in *Lamium*. Krawczyk et al. (2013) stated that the ITS region is inapplicable in *Lamium* identification; because of limitation of its use in polyploid plants, samples contaminated with fungal material or samples with partially degraded DNA. Instead, *psbA-trnH* cpDNA spacer has a high efficiency in the identification of the species (Krawczyk et al. 2013). However, in the nrITS phylogenetic tree, plant samples of each species belong to a distinct subgenus and section, were grouped together and formed separate cluster with respect to the cpDNA tree. Usage of the nrITS as a genetic marker for elucidating the molecular phylogeny of the species concerned, have been demonstrated in many studies (Zeng et al. 2003; Wang et al. 2005; Mao et al. 2007).

This is the first study on the use of ISSR markers for genetic diversity, species delimitation and determining genetic relationships among *Lamium* species in Iran. Mukherjee et al. (2013) showed that ISSR technique along with proper statistical tools could be successfully applied to assess the genetic diversity and phylogenetic analysis. Our results clearly demonstrated that the ISSR markers can be used in genetic diversity study as well as genetic identification of *Lamium*. Moreover, our results indicate very high efficiency of the ISSR markers in the identification and delimitation of *Lamium* species. Similar efficiency of the ISSR markers has also been reported by other authors (Ajibade et al. 2000; Galvan et al. 2003; Minaeifar et al. 2016; Esfandani-Bozchaloyi et al. 2017, 2018).

According to Mennema's subgeneric classification of *Lamium* (1989), *L. galeobdolon* have been placed in subgenus *Galeobdolon* (comprising species with glabrous anthers). *L. galeobdolon* has very distinct morphological characteristics from the genus *Lamium* (subequal, triangular, and acute lobes of the lower lip of the corolla); therefore, it has been included in a separate genus as *Lamiastrum* or *Galeobdolon*. However, Harley et al. (2004) and Govaerts et al. (2010) put *L. galeobdolon* in *Lamium*. Phylogenetic studies based on palynological (Abu-Asab and Cantino 1994), anatomical (Atalaya et al. 2016a) and molecular data (Bendiksby 2011a; Krawczyk 2013, 2014) of the genus *Lamium* revealed that this species should be put in this genus. Our result showed that position of *L. galeobdolon* within *Lamium* species varied between all trees; however, results from ISSR data support the inclusion of *L. galeobdolon* in the genus *Lamium*.

Cladistics analysis of the morphological characteristics (Ryding 2003) supported monophyly of *Lamium* subgenus *Lamium* (comprising species with hairy anthers; including sect. *Lamium*, sect. *Amplexicaule* and sect. *Lamiotypus*. Mennema 1989). However, monophyly of *Lamium* subgenus *Lamium* is neither controverted nor supported by molecular phylogenetic study (Bendiksby et al. 2011a). *L. garganicum* and *L. purpureum* are two members of sect. *Lamium* (comprises species with bracteoles and a straight corolla tube, Mennema 1989). In our morphological result, two prenominated species are not nested in the same clade,

because there are many significant morphological differences (leaf shape, length of calyx, ratio of calyx teeth length/ calyx tube length) between annual *L. purpureum* and *L. garganicum* which is perennial. For example, our morphological results showed that the calyx size ranges from 9–15 mm and the ratio of calyx teeth length to the tube length varied from 0.5–0.9 mm in *L. garganicum*, while they varied from 5-7 mm and 1.1-1.3 respectively, in *L. purpureum*. According to the recent phylogenetic studies presented by Bendiksby et al (2011a) and Krawczyk et al (2013), *L. purpureum* is not nested in the same clade as the members of *L. garganicum* complex, but rather they form different clades. Therefore, *Lamium* sect. *Lamium* supposed to be a polyphyletic section (Atalaya et al. 2016a). Moreover, our result obtained from ISSR data provided additional evidence useful in separating *L. purpureum* and *L. garganicum* in *Lamium* sect. *Lamium*. However, this finding is not supported by anatomical study on the genus (Atalaya et al. 2016a).

Close relationship between *L. album* and *L. tomentosum* (sect. *Lamiotypus.*, comprises species with bracteoles and a sigmoid corolla tube that is abruptly expanded and ventrally saccate, Mennema 1989) could be identified between all datasets. Monophyly of *L. album–L. tomentosum* group is strongly supported in the latest phylogenetic studies (Bendiksby et al. 2011a; Krawczyk et al. 2013).

Lamium amplexicaule var. *amplexicaule.* and *L. amplexicaule* var. *aleppicum* have been placed in sect. *Amplexicaule* Mennema. (includes species that lack bracteoles). As previously mentioned a close relationship between two varieties of *L. amplexicaule* could be identified between all results. However, the recent phylogenetic investigation provided by Bendiksby et al. (2011a) showed that *L. amplexicaule* is polyphyletic and that var. *aleppicum* does not group together with other *L. amplexicaule* varieties. Based on the molecular and morphological results, she suggested that *L. aleppicum* should be redefined as a species.

Genetic structure and gene flow

AMOVA analysis revealed that the species of *Lamium* are genetically differentiated. The low value of Nm (0.27) obtained based on ISSR data, indicated a few amounts of gene flow among the studied taxa. *Lamium* includes both diploid (2n=18) and tetraploid (4n=36, *L. confertum*, *L. galeobdolon* subsp. *argentatum* (Smejkal) J. Duvign. and subsp. *montanum* (Pers.) Hayek, and *L. hybridum* as *L. purpureum* var. *incisum*) taxa (Mennema 1989). Bendiksby et al (2011a) showed that the tetraploids taxa have allotetraploid origins. However, according to Mennema (1989), Iranian *Lamium* species comprises diploid taxa. We did not observe any intermediate forms in the morphological study, but morphological variability within each species did occur to some extent. This low degree of the genetic and morphological variability in *Lamium*, can help the species to local adaptations (Djamali et al. 2012). In addition, the broad use of natural resources to meet the needs of the developing human populations, deforestation and habitat fragmentation leads to reductions in the degree of gene flow among populations. This, in turn, increases the genetic differentiation among populations due to genetic drift (Setsuko et al. 2007; Hou and Lou 2011). Therefore, careful monitoring of genetic diversity and obtaining data on the population genetic structure is important for future conservation of *Lamium* genus in Iran.

The Mantel test produced significant correlation between geographical distance and genetic distance of the studied taxa that are called isolation by distance (IBD). Therefore, gene flow occurred between neighboring populations only.

In general, the present study revealed that combination of the morphological and ISSR data can delimit the species. Furthermore, our findings provided further useful support for the current molecular phylogeny and taxonomy of the genus *Lamium*. Moreover, the morphological studies of *Lamium*, based on complete sampling and usage of more variable molecular markers will be necessary to resolve infrageneric- level relationships.

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