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Biosystematic study of the genus *Lallemantia* (Lamiaceae): species delimitation and relationship

Fahimeh Koohdar*, Masoud Sheidai

Faculty of Biological Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran.

ABSTRACT *Lallemantia* (Lamiaceae) is a small genus with 5 species. In general, little biosystematics and molecular study has been performed on the genus *Lallemantia*. Moreover, the studies used only some of the species; none of them has considered all 5 species as a whole in one specific approach. Therefore, the species inter-relationship or nexus in the genus is not thoroughly probed. The present study investigated the molecular phylogeny and species relationship of all five species in the genus *Lallemantia*, using ribosomal protein L16 and the multilocus ISSR markers. It also compared their morphometric, anatomical and seed results. The species were efficaciously delimited by the morphological, anatomical and seed characters, as well as by ISSR and cpDNA markers. The PCA (Principal components analysis) plot of the species based upon the morphological characters, the MDS (Multidimensional Scaling) plot of the species based on the nutlet and anatomical characters, the NJ (neighbor joining) tree plot of ISSR data and the ML tree of cpDNA revealed closer affinity between *L. iberica* and *L. canescens* and *L. peltata* was placed at some distance from these species. The phylogenetic trees displayed monophyly of the genus *Lallemantia*. The Bayesian Evolutionary Analysis by Sampling Trees (BEAST) analysis unveiled that the studied *Lallemantia* species started to diverge about 25 million years ago.

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*Corresponding author
E-mail: f_koohdar@yahoo.com

Introduction

The genus *Lallemantia* (Lamiaceae) comprises of 5 species which are widely distributed in Afghanistan, China, India, Kazakhstan, Kyrgyzstan, Pakistan, Iran, Russia, Tajikistan, Turkmenistan, Uzbekistan, SW Asia and Europe, with the Caucasian region as the center of origin. All 5 *Lallemantia* species are found in Iran (Rechinger 1982). The species of *Lallemantia* are herbaceous plants, characterized by simple leaves; a thyrsoid, spike-like or oblong, often interrupted inflorescence; ovate to rotund or sometimes linear, aristate-toothed bracteoles; and oblong, trigonous, smooth and mucilaginous nutlets (Harley et al. 2004). The genus *Lallemantia* is closely related to *Dracocephalum* L., but it differs from *Dracocephalum* in having the upper lip of corolla with two internal longitudinal folds and distinctively 15-veins bracteoles which are aristate-dentate (Edmondson 1982). The *Lallemantia* species have been adopted as a source of food and medicine. For example, *L. iberica* (M.Bieb.) Fisch. & C.A. Mey. is consumed as an oil-seed plant in Iran and the USSR (Rivera-Nunez and Obonde-Gastro 1992; Dinç et al. 2009), furthermore, *L. royleana* (Benth.) Benth. seeds have significant antibacterial properties and are a good remedy for skin diseases

and the gastro-intestinal maladies (Mahmood et al. 2013).

Research on the nutlet in Lamiaceae has demonstrated that it is efficacious at various echelons of the taxonomic hierarchy to varying degrees. Specifically, pertained to myxocarpy (i.e. the phenomenon of mucilage production when the nutlets get wet), the anatomy of the pericarp in the family Lamiaceae has also been deemed an extremely useful taxonomic feature (Harley et al. 2004; Moon and Hong 2006; Dinç et al. 2009). The anatomical data as well as the nutlet surface sculpturing patterns have been proven to have a wide range of variation and diagnostic value for species recognition and taxonomy of Lamiaceae (e.g., Kaya et al. 2007; Alan et al. 2010; Kahraman et al. 2010a,b; Celep et al. 2014).

The molecular systematic investigations in the plants are carried out for different purposes: species delimitation, population divergence, species relationships, date of divergence determination, etc. (Broadhurst et al. 2004; Millar et al. 2011). Different molecular markers have been utilized to accomplish the tasks, e.g., AFLP (Amplified Fragments Length Polymorphism), SSRs (Simple Sequence Repeats), ISSRs (Inter-Simple Sequence Repeats). (Sheidai et al. 2012, 2013, 2014; Minaeifar et al. 2015). Both within and between the plant species, the technique of ISSR has been employed extensively for assessing the genetic as-

sociations. Apart from being easy and proffering a quick screen for the DNA polymorphism, ISSR only requires just infinitesimal amounts of DNA. Moreover, it does not need the information on the DNA template sequence. The molecular markers used in the phylogenetic investigations in plant groups are based on the investigation of nuclear ribosomal DNA and chloroplast genes and spacers (e.g., Olmstead and Palmer 1994; Zhang et al. 2015; Minaeifar et al. 2016). There is a consensus on the combination and simultaneous analysis of all the available data sets (Byrne 2003; Bakker et al. 2004). The ISSR and cpDNA information were used in the present study because these molecular markers are efficient for the species delimitation and the determination of the species relationships (Sheidai et al. 2013; Minaeifar et al. 2016).

Limited literature exists on the biosystematics studies on the genus *Lallemantia*. Dinç et al. (2009) reported the micro-morphological features of pollen, nutlet and trichome in three species of *L. iberica*, *L. canescens* (L.) Fisch. & C.A.Mey. and *L. peltata* (L.) Fisch. & C.A.Mey. occurring in Turkey. Alan et al. (2010) studied the anatomical facets in stem, leaf and root of the same species and Özcan et al. (2015) reported the chromosome number ($2n = 14$) for them.

Similarly, only a few biosystematic and molecular studies have been reported on the genus *Lallemantia* in Iran. Talebi and Rezakhanlou (2010) carried out the morphological analysis of all 5 species, while Dolatyari and Kamrani (2015) reported the karyotype features of four species. Jamzad et al. (2000) displayed a close kinship between *Nepeta* L. to *Lallemantia*, and their distinction from *Dracocephalum*. Additionally, according to Kamrani and Riyahi (2017) *Lallemantia* is a monophyletic genus.

In the present study, biosystematics investigation of the genus *Lallemantia* was performed using the morphological, anatomical and seed characters. Furthermore, the molecular analyses of all 5 species were carried out by the nuclear ISSR and the chloroplast DNA sequences to reveal the species delimitation and species relationships (Sheidai et al. 2013; Minaeifar et al. 2016).

Material and methods

Plant materials

Field investigations and collections were carried out in 2013–2015. For this study, 42 specimens of 5 species (*Lallemantia royleana*, *L. canescens*, *L. baldschuanica* Gontsch., *L. iberica* and *L. peltata*) were randomly collected from different geographic populations. Voucher specimens are deposited in Herbarium of Shahid Beheshti University (HSBU) (Table 1).

Table 1. *Lallemantia* species, their locality and voucher specimens.

Species	Locality	Voucher specimens	Accession number for cp-DNA study
<i>L. peltata</i>	Tehran	2014370	-
<i>L. peltata</i>	Alborz	2014371	MH453501
<i>L. canescens</i>	Qazvin	2014372	MH453498
<i>L. canescens</i>	West Azerbaijan	2014373	-
<i>L. canescens</i>	Zanjan	2014374	-
<i>L. baldschuanica</i>	Khorasan, Kalat	2014375	MH453499
<i>L. iberica</i>	Qazvin	2014376	-
<i>L. iberica</i>	West Azerbaijan	2014377	-
<i>L. iberica</i>	Zanjan	2014378	MH453500
<i>L. iberica</i>	Markazi	2014379	-
<i>L. iberica</i>	Kermanshah	2014380	-
<i>L. royleana</i>	Qazvin	2014381	-
<i>L. royleana</i>	Markazi	2014382	-
<i>L. royleana</i>	Mazandaran	2014383	-
<i>L. royleana</i>	Qom	2014384	-
<i>L. royleana</i>	Khorasan	2014385	MH453497
<i>L. royleana</i>	Tehran	2014386	-
<i>L. royleana</i>	Kerman	2014387	-
<i>L. royleana</i>	Shiraz	2014388	-

Morphometry and anatomy

Morphological characters studied displayed in Table 2.

For anatomical studies embedded materials were prepared as follows: three adult plants samples were excised and immediately fixed in formalin-acetic acid-alcohol (FAA) (formalin 5%, acetic acid 5% and 50% ethanol 90%) (Jensen 1962) for 48 to 72 h, and stored at 4 °C until sectioning. Samples then dehydrated in a graded ethanol series and embedded. After preparation of free transverse hand sections of the lamina and stem samples were washed

Table 2. Morphological characters studied in *Lallemantia* species.

Characters	The state of character
Plant habitat	1) annual; 2) biannual
Shape of bracteole	1) oval; 2) wedge; 3) circular
Plant height	
Length of basal leaf	
Width of basal leaf	
Length of petiole	
Length of stem leaf	
Width of stem leaf	
Length of bracteole	
Width of bracteole	
Length of calyx	
Length of corolla	
Length of nutlet	

Table 3. Anatomical characters studied in *Lallemantia* species.

No	Character
1	Thickness of epidermis in stem
2	Thickness of collenchymas in stem
3	Thickness of parenchyma in stem
4	Thickness of sclerenchyma in stem
5	Thickness of upper phloem in stem
6	Thickness of lower phloem in stem
7	Thickness of xylem in stem
8	Thickness of pith in stem
9	Thickness of epidermis in cross section of stem
10	Thickness of simple trichomes in stem
11	Thickness of glandular trichomes in stem
12	Thickness of upper epidermis in leaf
13	Thickness of lower epidermis in leaf
14	Thickness of collenchymas in leaf
15	Thickness of parenchyma in leaf
16	Thickness of mesophyll in leaf
17	Thickness of upper phloem in leaf
18	Thickness of lower phloem in leaf
19	Thickness of xylem in leaf
20	Thickness of simple trichomes in leaf

with distilled water and placed in 5% sodium hypochlorite solution for 20 min for clearing and rinsed with distilled water. The sections were stained with methylene blue and carmine and mounted on the slides using Canada balsam. Thin cut sections were observed under a microscope fitted with digital camera. Anatomical characters of stem and leaf were recorded in Table 3.

Nutlet

For scanning electron microscopy (SEM), nutlet samples were mounted on stubs using double-sided adhesive tape and coated with gold. The specimens were examined with a Phillips × L20 SEM. UTHSCSA Image Tool Version 3.0

was used to carry out required measurements. Nutlet characters were randomly measured and were used in phenetic analyses (Table 4).

Molecular studies

According to Sheidai et al. (2018) and Koohdar and Sheidai (2019), ISSR and cpDNA data were used for the purpose of studying the spices delimitation and relationships in *Lallemantia* genus. *Nepeta* L. (accession number: KT178247) and *Paonia* L. (accession number: KJ946020.1) were used as outgroups.

Data analyses

Species differences for morphological characters were investigated by ANOVA (Analysis of Variance) (Podani 2000). For multivariate morphological analyses, quantitative characters were divided into discrete groups and along with qualitative characters were coded as multistate characters. Grouping of the species was done by different ordination methods such MDS (Multidimensional scaling), and PCA (Principal components analysis) (Podani 2000). PCA was performed to identify the most variable morphological characters among the species studied (Podani 2000). PAST version 2.17 (Hammer et al. 2012) was used for multivariate analysis.

ANOVA was also performed to show anatomical difference among the species. Anatomical characters were first standardized (mean = 0, variance = 1) and used to establish Euclidean distance among pairs of taxa (Podani 2000). For grouping of the plant specimens, MDS was used (Podani 2000). PAST version 2.17 (Hammer et al. 2012) was used for multivariate analysis.

The ANOVA test was performed to show significant nutlet difference between the studied species. For grouping of the plant specimens, MDS was used. Nutlet data was standardized (mean = 0, variance = 1) for these analyses (Podani 2000). PCA was performed to identify the most variable nutlet characters among the populations studied.

Table 4. Nutlet characters studied in *Lallemantia* species.

Nutlets characters	The state of character
Nutlet shape	1) oblong; 2) oblong-triangular; 3) triangular
The apex shape of nutlet	1) obtuse; 2) acute
Nutlet surface	1) rounded cell arrangement; 2) verrucate; 3) verrucate- rugulate
Nutlet color	1) brown; 2) black
Length of nutlet	
Width of nutlet	
Length of wall sculpturing	
Width of wall sculpturing	
Length of cord	
Width of cord	

Table 5. Morphological data of *Lallemantia* species

Characters	<i>L. iberica</i>	<i>L. royleana</i>	<i>L. peltata</i>	<i>L. canescens</i>	<i>L. baldshuanica</i>
Plant habitat	Annual	Perennial	Annual	Annual	Annual
Shape of bracteole	Wedge	Elliptic	Rounded	Wedge	Elliptic
Plant height (cm)	20.5	26.3	24.9	9	20.62
Length of basal leaf (mm)	21.5	19.5	29.1	15.4	7.6
Width of basal leaf (mm)	13.5	8.2	15.9	11.8	4.5
Length of petiole of basal leaf (mm)	14	23.2	17	14.2	14
Length of stem leaf (mm)	16.5	28.4	31.1	7.6	26.8
Width of stem leaf (mm)	4	6.3	17.9	3.6	6.35
The length of bracteole (mm)	4.5	5	12.3	5.6	4.65
Width of bracteole (mm)	1	3.2	12.2	2.3	2.4
Length of calyx (mm)	7.5	18.7	16.2	6	10.3
Length of corolla (mm)	10.5	28.7	16.95	9.43	12.15
Length of nutlet (mm)	3.25	3.34	2.97	3	3.35

For ISSR analyses binary characters (presence = 1, absence = 0) were used to encode ISSR bands. NJ (neighbor joining) (Saitou and Nei 1987; Podani 2000) was used for grouping. Paleontological statistics (PAST) ver. 2.17 was used for analysis (Hammer et al. 2012).

In case of cp-DNA sequences analyses, several phylogenetic methods were applied. 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). 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 (Minaeifar et al. 2016). Moreover, Hasegawa, Kishino and Yano model (HKY)

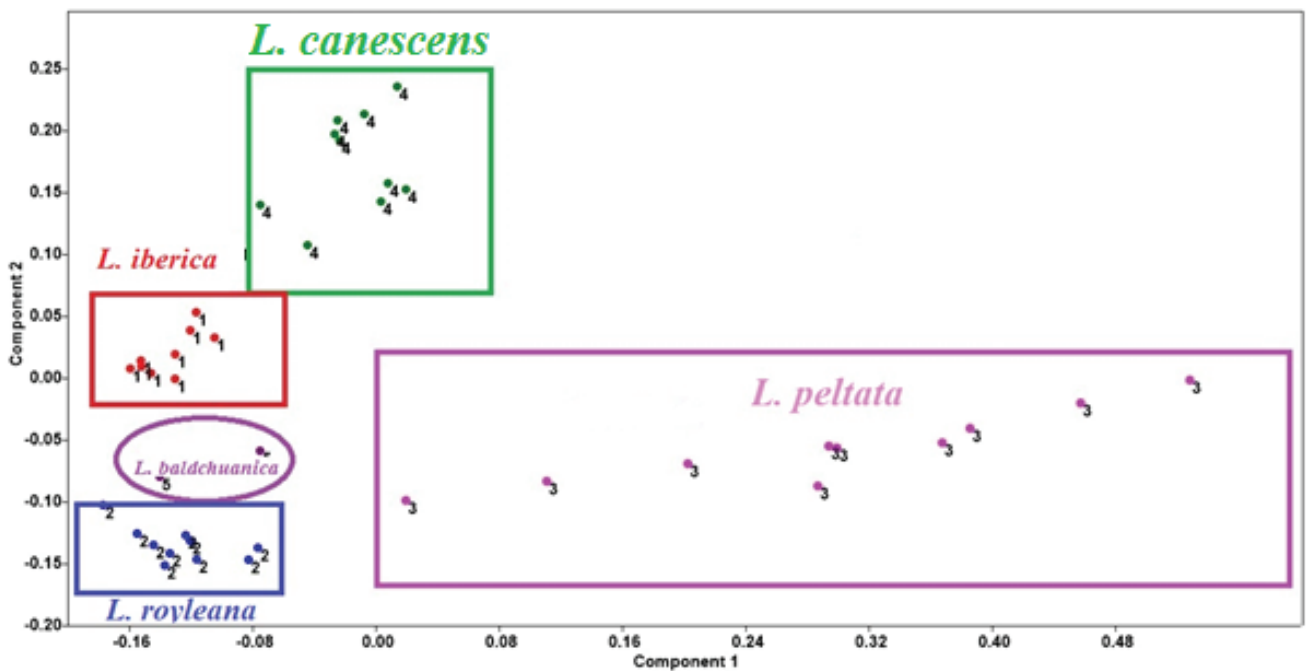


Figure 1. PCA plot of morphological characters in *Lallemantia* species.

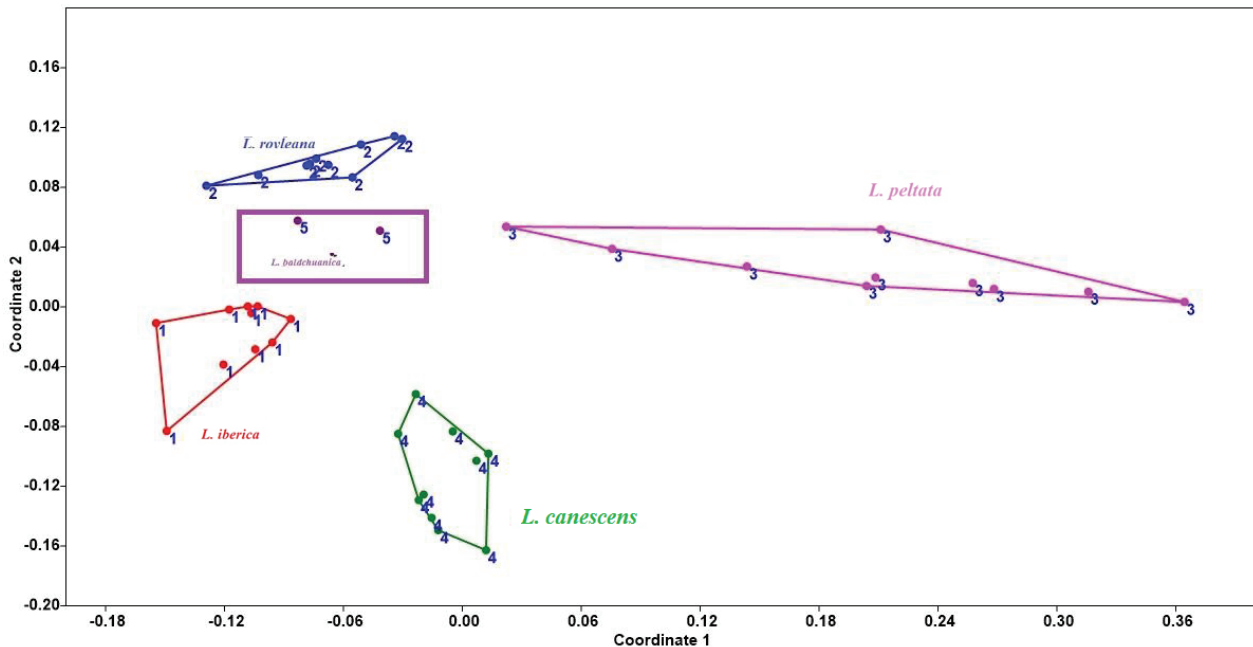


Figure 2. MDS plot of morphological characters in *Lallemantia* species.

was the best substitution model identified by model test as implemented in MEGA 5 (Tamura et al. 2011).

BEAST v1.6.1 (Drummond et al. 2010a; Drummond et al. 2010b) was used for the Bayesian Markov chain Monte Carlo (MCMC) inferred analyses of the nucleotide sequence data (Rambaut and Drummond 2007). *Nepeta* L. (accession number: KT178247) and *Paeonia* L. (accession number: KJ946020.1) were used as out groups.

BEAUti (Bayesian Evolutionary Analysis Utility version) v1.6.1 (Drummond et al. 2010a,b) 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,b) and can be regarded as explaining the net speciation rate (Nee 2006). For the MCMC analysis, the chain length was 10000000. After discarding 100 trees representing the burn-in, 10000 trees were used for the analysis. The BEAUti xml file was run in BEAST v1.6.1 (Drummond et al. 2010a, 2010b). Because no fossils were available for the studied species, we assumed a rate of evolution of the plastid sequence ($\mu = 1.0 \times 10^{-9} \text{ s}^{-1} \text{ year}^{-1}$; 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.

Results

Morphometry

Details of mean of morphological characteristics in five studied species are provided in Table 5.

The ANOVA test showed significant difference ($p < 0.05$) for quantitative morphological characters among *Lallemantia* species. Different ordination methods like PCA and MDS produced similar results. PCA and MDS plots of morphological characters (Figs. 1 and 2) separated the studied species from each other. In this plot, *L. iberica* and *L. canescens* were placed close to each other, while *L. peltata* was placed far from the others due to difference in characters width of stem leaf, the length of bracteole and width of bracteole.

PCA analysis revealed that the first 3 components comprised about 88% of total morphological variability. In the first PCA components with about 57% of total variation, characters like length of basal leaf, width of stem leaf, length of bracteole, width of bracteole, shape of bracteole showed the highest positive correlation (> 0.80).

Table 6. Anatomical data of *Lallemantia* species.

Characters	<i>L. canescens</i>	<i>L. peltata</i>	<i>L. iberica</i>	<i>L. royleana</i>
Thickness of epidermis in stem	28	32.2	25.8	28.6
Thickness of collenchyma in stem	181.6	238.1	224.2	126.9
Thickness of parenchyma in stem	67.9	91.5	67.8	48.6
Thickness of sclerenchyma in stem	27.3	37.7	33	30
Thickness of upper phloem in stem	38.2	50.8	56.6	39.1
Thickness of lower phloem in stem	168.7	180.07	179.5	156.9
Thickness of xylem in stem	67.4	87.02	63.4	57.8
Thickness of pith in stem	1596.3	993.42	1196.9	937.4
Thickness of epidermis in cross section of stem	2041.6	1511.9	1939.8	1362.4
Thickness of simple trichomes in stem	107.8	108.6	104.8	118.6
Thickness of glandular trichomes in stem	29.1	27.5	40.08	35.8
Thickness of upper epidermis in leaf	20.0	20.8	22.3	17.1
Thickness of lower epidermis in leaf	26.6	17.75	21.00	27.3
Thickness of collenchyma in leaf	42.7	37.4	41.2	60.1
Thickness of parenchyma in leaf	92.6	60.8	86.0	94.7
Thickness of mesophyll in leaf	195.5	171.6	188.8	272.5
Thickness of upper phloem in leaf	29.7	36.9	34.9	37.5
Thickness of lower phloem in leaf	31.67	29.50	26.40	34.00
Thickness of xylem in leaf	85.0	47.7	38.9	60.1
Thickness of simple trichomes in leaf	75.00	64.50	53.40	79.67

The length of corolla, shape of inflorescence, life-history strategy, showed the highest positive correlation (> 0.80) with the second PCA component. These characters may be used in taxonomy of the genus and delimiting *Lallemantia* species.

Anatomy

The detailed descriptions of the anatomical features in the studied species are displayed in Table 6. The representative anatomy of each species is displayed in Figures 3 and 4.

The stems in the cross section have a square form with pronounced angles and are covered with a one-layered epidermis. Collenchyma is single layered among the angles, but 5-10 layers of collenchyma are observed below the epidermis at the angles. Phloem and xylem were regular cylinders. The highest epidermis (32.2 μm), collenchyma (238.1 μm), parenchyma (91.5 μm), sclerenchyma (37.7 μm),

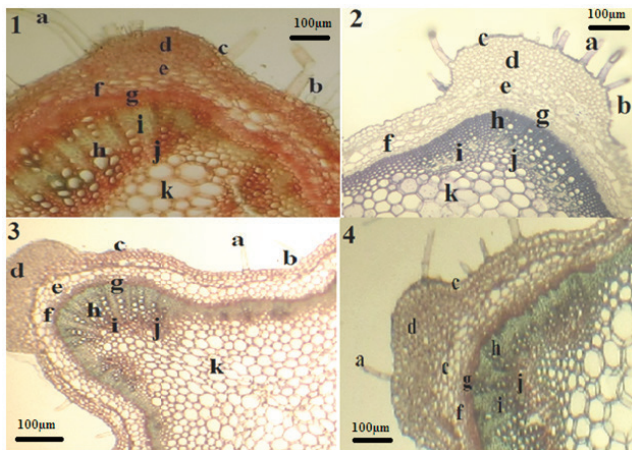


Figure 3. Appearance of stem in *Lallemantia* species.

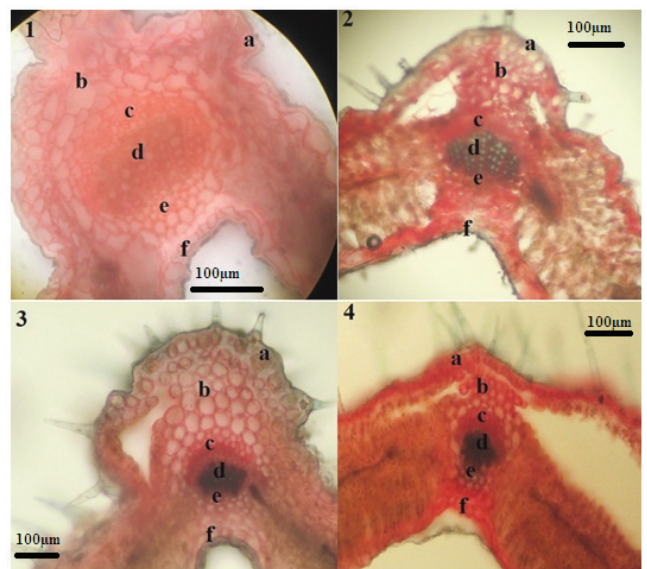


Figure 4. Appearance of leaf in *Lallemantia* species.

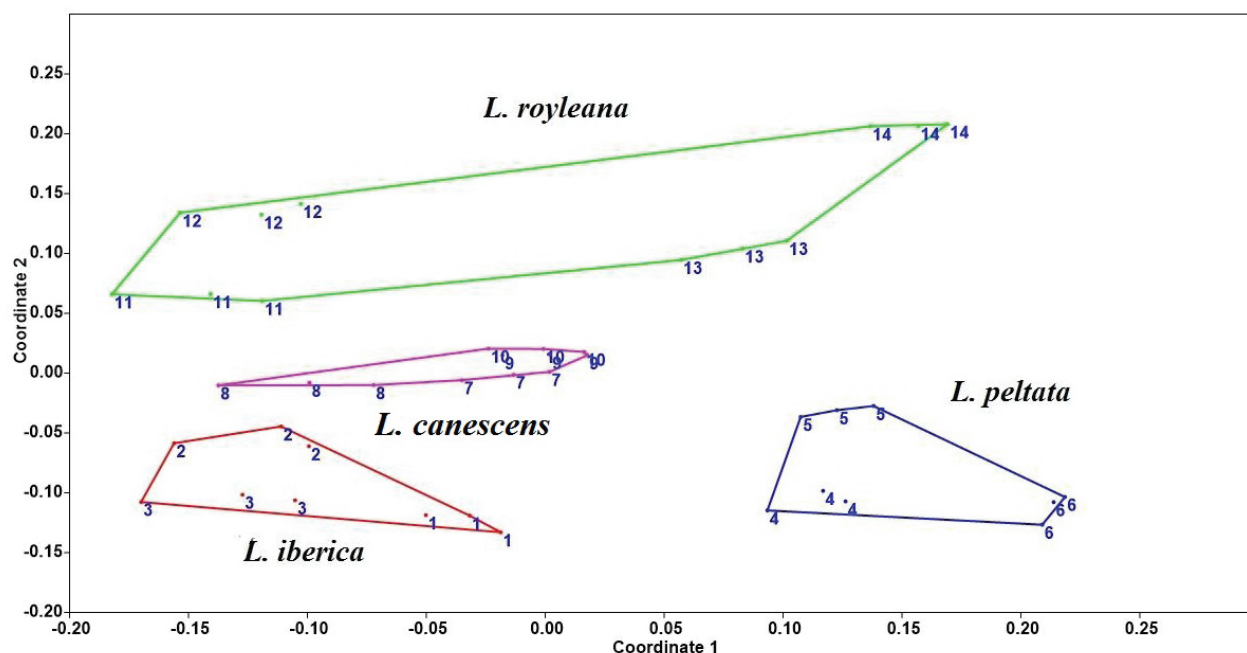


Figure 5. MDS plot of anatomical characters in *Lallemantia* species.

xylem ($180.07 \mu\text{m}$) and lower phloem ($87.02 \mu\text{m}$) length in stem was observed in *L. peltata* while *L. canescens* had the highest value length of pith in stem ($1596.3 \mu\text{m}$), length of stem in transverse transects ($2041.6 \mu\text{m}$) and width of stem in transverse transects ($1881.3 \mu\text{m}$).

All of the leaves in the sections were bifacial (dorsiventral and amphistomatic mesophyll) type and were composed of one layered epidermis. The highest length of the lower epidermis ($27.3 \mu\text{m}$), collenchyma ($60.1 \mu\text{m}$), parenchyma ($94.7 \mu\text{m}$), mesophyll ($272.5 \mu\text{m}$), upper phloem ($37.5 \mu\text{m}$) and lower phloem ($34.00 \mu\text{m}$) in leaf was observed in *L. royleana*. The ANOVA test exhibited significant difference ($p < 0.05$) for the quantitative anatomical characters among *Lallemantia* species.

The MDS plot of the anatomical characters (Fig. 5) separated the scrutinized species from one another. In this plot, *L. iberica* and *L. canescens* were placed close to each other because of the traits such as the number of layers in collenchyma and parenchyma in stem; *L. peltata*, however due to its shape of collenchyma and *L. royleana* were placed far from the others.

Nutlet

The details for mean of the nutlet characteristics in 5 studied species are depicted in Table 7. The SEM micrographs of nutlets are presented in Figure 6. The shapes of nutlets are oblong in *L. royleana* and *L. baldshuanica*, triangular in *L. peltata* and oblong-triangular in *L. canescens* and *L.*

Table 7. Nutlet data of *Lallemantia* species.

Characters	<i>L. iberica</i>	<i>L. royleana</i>	<i>L. peltata</i>	<i>L. canescens</i>	<i>L. baldshuanica</i>
Nutlet shape	Oblong	Oblong-triangular	Triangular	Oblong	Oblong triangular
The apex shape of nutlet	Obtuse	Acute	Acute	Obtuse	Acute
Nutlet surface	Rounded cell arrangement	Verrucate	Verrucate-rugulate	Rounded cell arrangement	Verrucate
Color	Black	Black	Black	Black	Black
Length of nutlet (μm)	3554	2664	2538	3448	3021
Width of nutlet (μm)	1115.2	1400	959.48	978	1650
Length of cord nutlet (μm)	202.69	865	645.76	219	951.29
Width of cord nutlet (μm)	269.91	721	351.81	338	897.2
Length of wall sculpturing (μm)	47.74	52.32	40.86	72.59	56.75
Width of wall sculpturing (μm)	41.42	41.48	27.97	28.4	26.28

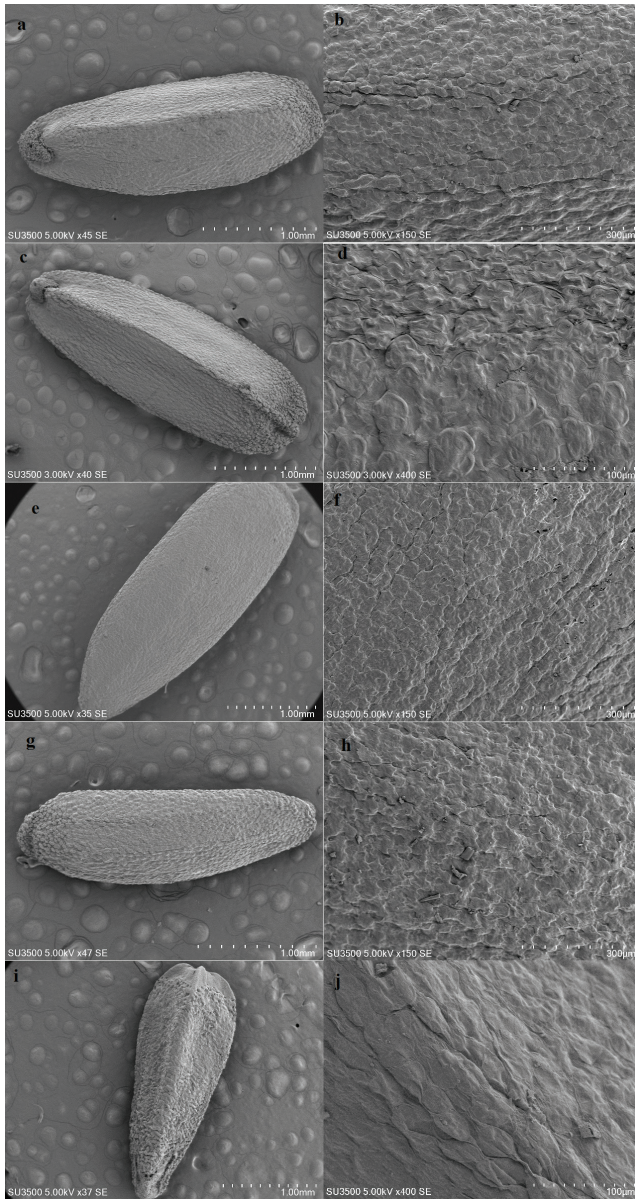


Figure 6. General appearance and surface details of nutlets of *Lallelantia* species. a, b: *L. royleana*; c, d: *L. baldshuanica*; e, f: *L. canescens*; g, h: *L. peltata*; i, j: *L. iberica*.

iberica. The colors of nutlet in all the examined species alter from brown to black. The apex of nutlet is obtuse or acute. The nutlet surface is verrucate-rugulate in *L. peltata*, verrucate in *L. iberica* and *L. canescens* and rounded cell arrangement in *L. royleana* and *L. baldshuanica*. The highest length of nutlet (3.55 μm), width of nutlet (1.64 μm), length of cord nutlet (951.29 μm), width of cord nutlet (897.2) were observed in *L. iberica* while *L. peltata* had the highest value length of wall sculpturing (1596.3 μm). The highest length of wall sculpturing was observed

in *L. royleana* (72.59 μm).

ANOVA revealed significant difference in the nutlet quantitative characters. The MDS plot (Fig.7) of nutlet showed that while *L. iberica*, *L. canescens*, *L. baldshuanica* and *L. royleana* were placed close to one another, *L. peltata* was placed far from the others.

ISSR analyses

Almost all the ISSR primers produced bands were used and finally a data matrix of 42×104 was formed for further analysis. The highest number of the bands was observed in *L. royleana* (53) and *L. peltata* (45), while *L. baldshuanica* had the lowest value (21). *L. baldshuanica*, *L. iberica* and *L. canescens* had 3, 1 and 1 unique bands, respectively, the other bands were common in the studied samples.

The NJ tree of ISSR data (Fig. 8) grouped the specimens of each species together in a single cluster, separated from the other species. This means that ISSR molecular markers are of taxonomic value and can delimit the *Lallelantia* species. The NJ tree showed closer genetic affinity between *L. iberica* and *L. canescens* while *L. peltata* differs from the others.

Species relationship based on cpDNA sequences

After Cp-DNA multiple sequence alignment by MULTIPLE Sequence Comparison by Log-Expectation (MUSCLE) and curing the sequences, 385 sequences remained for phylogenetic tree construction. Out of these 91 sequences was parsimony informative. Different phylogenetic analyses produced similar results. Therefore, only maximum likelihood (ML) and Bayesian phylogenetic tree are presented (Figs. 9a and b). In both phylogenetic trees, *L. canescens*, and *L. iberica* were placed close to each other in a single clade. In both analyses, the out-group was separated from the other species studied.

Species divergence time

In order to choose the type of the nucleotide evolution in BEAST, the molecular clock test was performed by comparing the ML value for the given topology with and without the molecular clock constraints under the Kimura 2-parameter model (+G). The null hypothesis of the equal evolutionary rate throughout the tree was rejected at a 5% significance level ($P = 0.016$). Hence, the relaxed molecular clock was utilized in further analysis. The Bayesian tree (phylogram) of BEAST is provided in Fig. 9c.

The oldest node of *Lallelantia* appeared in Iran about 25 Mya, followed by the node that led to the formation of *L. royleana* and *L. peltata* in about 23 Mya. The elicited tree dates back the *L. baldshuanica* appearance to about 15 Mya. However, active radiation occurred from 12-13 Mya.

Discussion

The morphometric, anatomical data, nutlet features and molecular data which were obtained after this study could differentiate the *Lallemantia* species efficaciously. Dinç et al. (2009) gives confidence to the above statement by their previous study. The present study revealed that the gleaned species relationship based on the molecular data almost agrees with the morphological, anatomical and nutlet micro-morphological data. The only difference between these two types of data is found in *L. baldshuanica*. The species relationship presented here is supported also by the morphological studies of Talebi and Rezakhanlou (2010).

The nutlet surface sculpturing patterns as seen by SEM demonstrate a vast array of variation and have diagnostic value for species recognition in the tribe Mentheae (Jamzad et al. 2000; Moon and Hong 2006; Kaya and Baser 2007). Nevertheless, in *Lallemantia*, external nutlet characters and nutlet surface sculpturing patterns are clearly a good indicator for the interspecific classification.

From the findings obtained from the previous studies, it can be concluded that the anatomical characteristics are as useful as taxonomic characters at the species level in Lamiaceae (Pădure 2003). The stems of it in cross section have a square form with pronounced angles and are covered with a one-layered epidermis. Collenchyma is single layered among the angles, but 5-10 layers of collenchyma are observed below the epidermis at the angles. Phloem

and xylem were regular cylinders. The highest epidermis, collenchyma, parenchyma, sclerenchyma, xylem, and lower phloem length in stem was observed in *L. peltata* while *L. canescens* had the highest value length of pith in stem, length of stem in transverse transects and width of stem in transverse (1881.3 μm). All of the leaves in the sections were bifacial (dorsiventral and amphistomatic mesophyll) type and were composed of one layered epidermis. The highest length of lower epidermis, collenchyma, parenchyma, mesophyll, upper phloem and lower phloem in leaf were observed in *L. royleana*.

According to our BEAST results, the node leading to the formation of *royleana* appeared in Iran about 23 Mya; therefore, it can be stated that *L. royleana* appeared first in Khorasan province and then due to population genetic divergence, *L. baldshuanica* was formed about 15 Mya in Khorasan province.

L. baldshuanica showed close genetic affinity with *L. iberica* and *L. canescens*. These two latter species occur in Zanjan and Qazvin provinces that are in the vicinity of Tehran in the North-West part of Iran. It may also be safe to conclude that the process of speciation and migration had occurred from East towards West of Iran; the formation of *L. iberica* and *L. canescens* seems to have taken place in these regions, too.

High physiological heterogeneity is considered to be a major influence on the high floral diversity (Grimm and Denk 2014). One component of this heterogeneity is a series of mountains extending from West to the

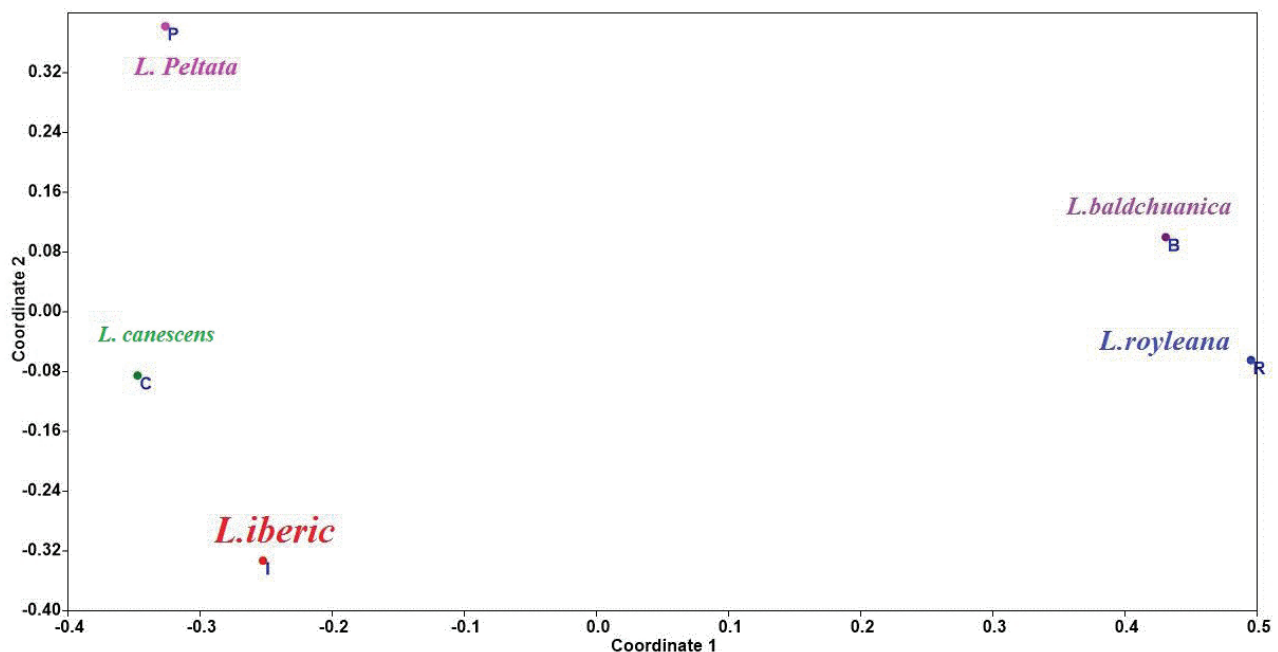


Figure 7. MDS plot of seed characters in *Lallemantia* species.

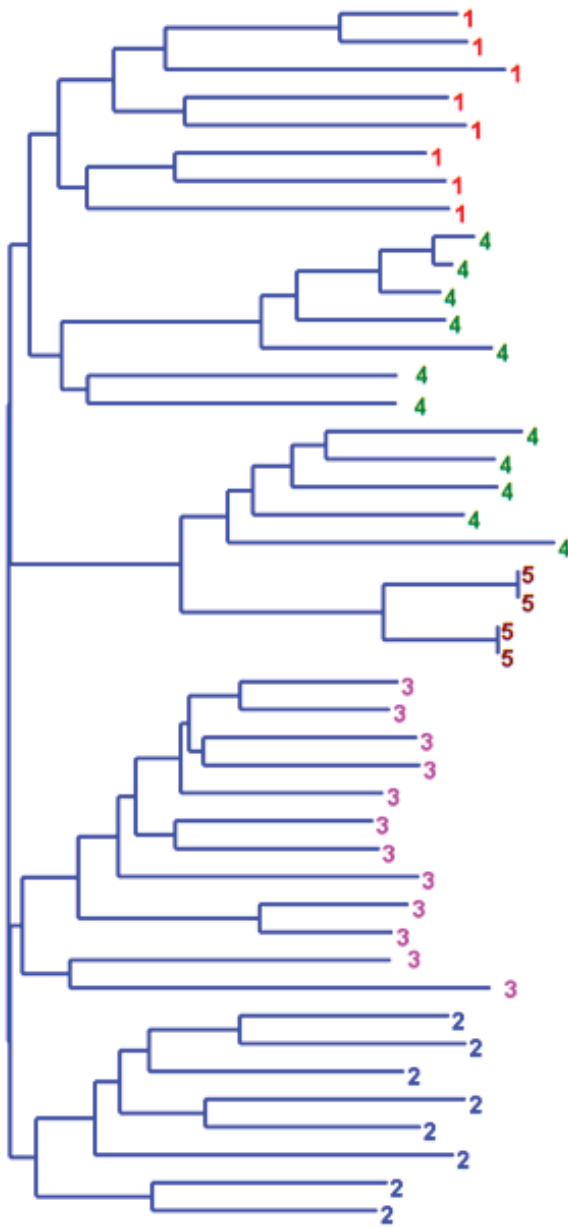


Figure 8. Neighbor joining tree of ISSR data in the studied *Lallelantia* species. 1: *L. canescens*; 2: *L. royleana*; 3: *L. peltata*; 4: *L. iberica*; 5: *L. baldshuanica*.

Northeast of Iran where *Lallelantia* species are growing. The *Lallelantia* species are distributed in different ecological regions with altitude of -20 to 1500 meters high from sea level in Iran. This extensive heterogeneity may bring about some degree of genetic differentiation and accelerate the speciation process witnessed in Western Iran. The active speciation during 12-13 Mya in these mountainous regions may be due to their reactions to the Pleistocene glaciations.

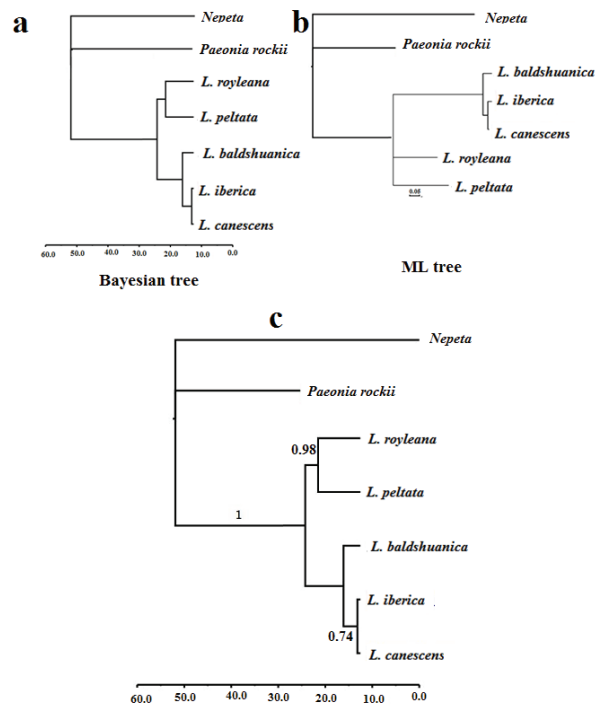


Figure 9. a: Bayesian phylogenetic tree; b: Maximum likelihood phylogenetic tree of *Lallelantia* species based on the cp-DNA dataset (rpL16). Values of all branches are above 90% bootstrap values. Scale: million years ago. c: Phylogenetic tree and chronogram using BEAST Bayesian inference based on the cp-DNA dataset (rpL16). Values at left of nodes on the tree are bootstrap support above, and posterior probability below. At the right of nodes are the estimated dating values and their 95% HPD from BEAST..

The palaeogeographic reconstructions signal that the area corresponding to the Southern part of the present Alborz Mountains was not depositional/ erosional and most likely formed a mountain range since the Late Cretaceous time at ca. 65 Ma. Unlike the southern section of Alborz, however, the northern section is a geologically younger area which was subsiding under the Paratethys Ocean until the middle Miocene about 10-15 Mya (Berberian and King 1981). This is in agreement with the earlier appearance of *L. peltata* in Alborz mountain than the other studied species appeared in the Western region of Zagros mountain, like *L. iberica* and *L. canescens* appearing most recently in Zanjan and Qazvin. Kuhle (2008) in his glacio-geomorphological study of Iran estimated the snowline descent to be approximately 1400 to 1600 m with temperature decline of 11 to 15 °C, and the occurrence of Pleistocene glaciation in Zagros.

Drew and Systma (2012) studied the biogeography of the tribe Mentheae by using ITS molecular data and *L. canescens* as the representative of *Lallelantia*. They took the Miocene period (about 11 Mya) to be the probable divergence time for *L. canescens*; this finding was in ac-

cordance with the present paper.

In conclusion, the present study revealed the taxonomic implication of the multiple data sets in *Lallemantia* species delimitation and also uncovered the species relationship. The monophyly of the genus *Lallemantia* was shown through the phylogenetic trees.

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