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Systematic Morphological and Molecular Studies in Genus *Lonicera* L. (Caprifoliaceae)

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ABSTRACT

Genus *Lonicera* has members that have opposite, narrowly elliptic to obovate leaves and yellow-white, red, or purple-red corollas together with capitate stigmas and undulated calyxes. According to Wendelbo (1965) in Flora Iranica, the 19 members of *Lonicera* are classified into two subgenera, *i.e*., *Lonicera* and *Chamaecerasus*, and three sections, *i.e*., *Isika*, *Isoxylosteum,* and *Coeloxylosteum.* The four studied species belong to subgenus *Chamaecerasus* and sections *Isika* and *Coeloxylosteum*. The taxonomy and phylogeny of this genus is highly complicated and controversial. The present study was done by the use of phenetic analyses of morphology together with Bayesian analyses of molecular data (ITS sequences) to illustrate the species relationships, taxonomic classification, and monophyly versus paraphyly of the species in genus *Lonicera.* We used seven *Lonicera* species for molecular studies, for which nrDNA-ITS sequences were newly obtained. Successive reweighting with rescaled consistency index was used to conduct the molecular examination, which showed close similarities among the results of maximum likelihood, maximum parsimony, and Bayesian methods based on the ITS dataset were observed. This study showed that in general, it is possible to differentiate the species via morphological features. Phylogenetic relationships within *Lonicera* were revealed, and ITS-based phylogenetic trees and morphological characters were in agreement.

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Introduction

Biosystematics investigations in plants comprise different tasks including population divergence, species delineation, date of divergence, and species relationships. Such data are not complete for the majority of plant groups and one or a few of these types of investigations have been performed in them. This holds true for the genus *Lonicera* (Caprifoliaceae)**.**

Above 200 species across the globe belong to *Lonicera* L. (Caprifoliaceae) family (Mabberley, 2008), among which 19 species are found in the Flora Iranica region (Wendelbo, 1965). The genus is mainly scattered in the northern hemisphere from temperate to subtropical places: Europe, Russia, East Asia, and North America (Hsu and Wang, 1988; Mabberley, 2008). Nine species scattered across the north, northwest, and northeast of the country represent genus *Lonicera* in the flora of Iran (Ghahremaninejad and Ezazi, 2009). Some species are medicinal herbs (Zeng *et al.*, 2017). Buds and flowers of *Lonicera* are dried and known as Flos Lonicera, which has been a known herb in Chinese traditional medicine for above 1500 years (Li *et al.*, 2015) and applied for the treatment of diabetes mellitus, arthritis, viral infections, and fever (Shang *et al.*, 2011; LI *et al.*, 2015). These plants are erect shrubs, occasionally small trees. Genus *Lonicera* has members with opposite, narrowly elliptic to obovate leaves and yellow-white, red, or purple-red corollas together with

capitate stigmas (Judd *et al.*, 2008) and undulated calyxes. Wendelbo (1965) in Flora Iranica divided 19 members of *Lonicera* into two subgenera, *i.e*., *Lonicera* and *Chamaecerasus*, and three sections, *i.e*., Isika, *Isoxylosteum,* and *Coeloxylosteum.* The four species under study are members of subgenus *Chamaecerasus* and sections *Isika* and *Coeloxylosteum*.

Phylogenetic and speciation studies have led to molecular data (Osaloo *et al.*, 2003; Osaloo *et al.*, 2005), based on which supportive and extra criteria could be developed to systemically classify species of interest; a task that has been done so far only based on morphological features (Chase *et al.*, 1993). Internal transcribed spacers (ITS) are regions of 18S-5.8 S-26S nuclear ribosomal cistron (Baldwin *et al.*, 1995) and contain required signals for the processing of the rRNA transcripts (Baldwin, 1992; Baldwin *et al.*, 1995). ITS has often been employed to infer phylogeny in plants at the generic and infra-generic levels (*e.g*., Baldwin, 1992; Baldwin *et al.*, 1995; Osaloo *et al.*, 2003; Osaloo *et al.*, 2005). Theis *et al.* (2008) used nuclear and chloroplast DNA sequences to study the phylogenetic of *Lonicera* (*Dipsacales*) and *Caprifolieae*. Their analysis indicated monophyly in *Lonicera* and highlighted instances of homoplasy in several morphological characters. Molecular phylogenetic analysis of *Lonicera* in Japan was conducted by Nakaji *et al.* (2015) based on chloroplast DNA sequence. The results show that the proposal of Hara (1983) for circumscribing higher taxonomy groups for the Japanese species of *Lonicera* is suitable. It is well-known that *Lonicera* is taxonomically complex, which is due to morphological characters overlapping. Molecular data and advanced bioinformatics analyses have been extensively used to answer the existing questions on mechanisms of plant groups, species relationships, and their mode of divergence. The Molecular data are gathered from various molecular markers and gene sequences. Multilocus molecular markers are non-selective (neutral) in nature and comprise numerous kinds: for example, SSRs (simple sequence repeats), ISSRs (inter-simple sequence repeats), AFLP (amplified fragments length polymorphism), and retrotransposon (REMAP) (Bozchaloyi *et al*., 2017a; Bozchaloyi *et al*., 2017b). Nuclear ribosomal DNA and chloroplast genes and spacers are the main gene sequences most often used in plant molecular systematics and phylogenetic investigations (Bozchaloyi *et al*., 2017c; Bozchaloyi *et al*., 2017d; Bozchaloyi *et al*., 2018). Combining and simultaneously analyzing all available datasets has a wide acceptance (Bakker *et al.*, 2004). There has been no detailed molecular systematic research on genus *Lonicera* in Iran. Furthermore, in Iran, the number of species that have overlapping scattering areas and can produce interspecific hybrids is small. Therefore, the present research was carried out to clarify the relationships of native *Lonicera* species of Iran.

Materials and Methods

Plant materials

For morphometric studies (phonetic analyses), we used 70 plant specimens of seven *Lonicera* species growing in Iran (Table 1, Fig.1) and for nrDNA ITS phylogenetic tree, 9 species (Two species of *Leycesteria* (*L. formosa* wall. and *L. crocothyrsos* Airy Shaw) were selected as outgroups. Voucher specimens were placed in the Herbarium of Islamic Azad University of Tehran (IAUNT). Here, the sequences of NrDNA-ITS were obtained for seven species, with the remaining sequences provided from GenBank. The appendix contains data regarding voucher specimens and previous sequences that have been published.

Morphological studies

Morphometry was conducted with 4-5 specimens from each species. Totally, 52 morphological features were investigated, of which 23 were qualitative and 29 were quantitative, as can be seen in Table 2. After standardizing the obtained results with mean= 0 and variance = 1, they were employed for estimating Euclidean distance for ordination and clustering analysis (Podani, 2000).

ITS sequences

The amplification of the nrDNA-ITS region was conducted with both ITS4 and ITS5 as primers (White *et al*. 1990; Taberlet *et al.* 1991) (Table 3). PCR reactions took place in a 25-µl solution consisting of 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 1 U of *Taq* DNA polymerase (Bioron, Germany). The procedure below was followed using a Techne thermocycler (Germany) to conduct the amplification reactions: a 5-min initial denaturation step at 94 °C, and then 35 cycles for 1 min at 94 °C, 45 s at 57 °C, and 2 min at 72 °C. A final extension step for 7-10 min at 72 °C was used to complete the reaction. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. A 100-bp molecular size ladder (Fermentas, Germany) was utilized to estimate the fragment size. White *et al*. (1990) and Taberlet *et al.* (1991) reported using universal primers to amplify ITS and *trn*L-F regions, respectively, in flowering plants, as can be seen in Table 3.

Table 1. *Lonicera* species and populations, their localities, and voucher numbers.

Sp.	Locality	Latitude	Longitude	Altitude (m)	Voucher no.
Lonicera floribunda Boiss. and Buhse	Mazandaran, Chalus, Valiabad	38 ˚ 52393"	47 ˚ 25 92"	1133	IAUH 201677
	Mazandaran, Noshahr, Kajoor	38 ˚ 52353"	47 ˚ 27 92"	1143	IAUH 201678
L. iberica M. Bieb.	Golestan, Jahan Nama	38 ˚ 52'373	47 ˚ 23' 92"	1144	IAUH 201679
	Tehran, Firuzkuh Road, Gaduk	38 ˚ 52'353	47 ˚ 27' 92"	1143	IAUH 201680
	Mazandaran, Kelardasht Dalir	38 ˚ 52'393	47 ˚ 25' 92"	1137	IAUH 201681
	Semnan, Mehdishahr, Fenisk Jungle	38 ˚ 51' 51"	47 ˚ 02' 28"	1155	IAUH 201682
L. nummulariifolia Jaub. et Spach	Semnan, Tange parvar	38 ˚ 52'373	47 ˚ 23' 92"	1144	IAUH 201683
L. bracteolaris Boiss. and Buhse	Semnan, Shahrud, Abr Forest	38 ˚ 52'353	47 ˚ 27' 92"	1143	IAUH 201686
L. caucasica	Mazandaran, Chalus, Pole Zangoole	37 ˚ 09 55"	49 ˚ 55 49 "	32	IAUH 201689
L. hypoleuca Decne.	Hormozgan, Bandar Abbas, Siyahu	370702.32	49 ˚ 4432.6	48	IAUH 201690
L. korolkowii Stapf	Semnan, Mehdishahr, Sheli	38 ˚ 52'373	47 ˚ 23' 92"	1144	IAUH 201695

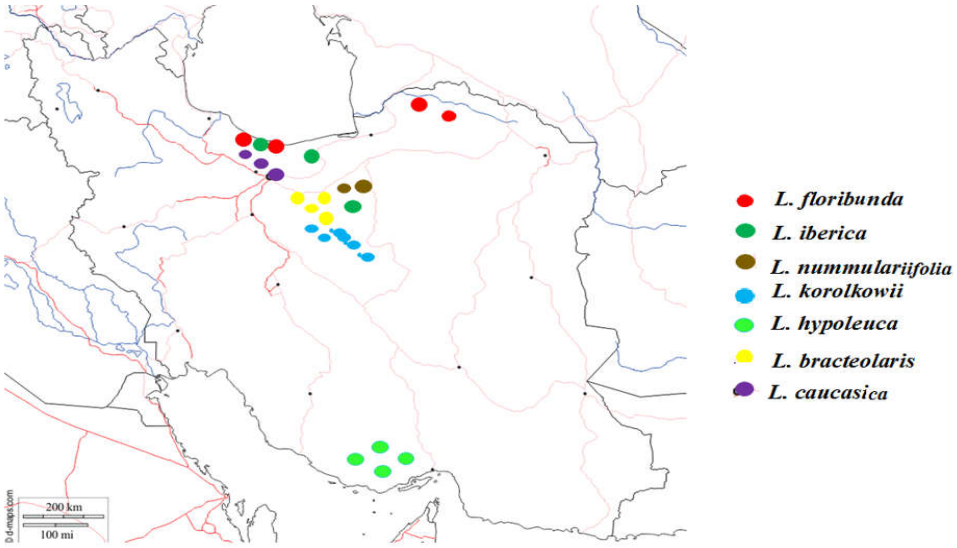

Fig. 1. Distribution map of studied species.

Data analysis

Morphological studies

We used phenetic analysis for morphological data. To classify the plant specimens, an unweighted pair group method with arithmetic mean (UPGMA), Ward's minimum variance, and principal coordinate analysis (PCoA) plot were used (Podani, 2000). All morphological characters contained 23 qualitative and 29 quantitative were used (Table 2). For identifying morphological features with the greatest variation among the populations of understudy, principal components analysis (PCA) biplot was employed (Podani, 2000). Maximum parsimony (MP was used for cladistics analysis, followed by bootstrapping (100 times). To conduct these analyses, PAST software v. 2.17 (Hammer *et al.*, 2012) and PAUP (Swofford, 2002) were employed. Both qualitative and quantitative features were applied for maximum parsimony

analyses. For this purpose, quantitative features were coded.

Table 2. Morphological characters in studied species.

No	Characters	No	Characters	No	Characters
1	Plant height (mm)	19	Petal length / Petal width (mm)	36	Pedicel length (mm)
2	Length of stem leaves petiole (mm)	20	Leaf hair density	37	Peduncle length (mm)
3	Length of stem leaves (mm)	21	Calyx apex	38	Stem hair density
4	Width of stem leaves (mm)	22	State of stem strength	39	Style length (mm)
5	Length of stem leaves / Width of stem leaves(mm)	23	State of stem branches	40	Stamen filament length (mm)
6	Width of stem leaves/ Length of stem leaves (mm)	24	Leave shape	41	Fruit length (mm)
7	Number of segment stem leaves (mm)	25	Phyllotaxy	42	Number of flowers per inflorescence
8	Length of basal leaves petiole (mm)	26	Petioles hair density	43	Bract shape
9	Length of basal leaves (mm)	27	Sepale hair	44	Stipules shape
10	Width of basal leaves (mm)	28	Sepale hair density	45	Bract and Stipules hair density
11	Length of basal leaves / Width of basal leaves (mm)	29	Peduncle and pedicel hair	46	Bract and Stipules hair
12	Width of basal leaves / Length of basal leaves (mm)	30	Stipules length (mm)	47	The shape of segments cauline leaves
13	Number of segment basal leaves	31	Stipules width (mm)	48	Shape of calyx
14	Calyx length (mm)	32	Stipules length/Stipules width (mm)	49	Leaf tips
15	Calyx width (mm)	33	Bract length (mm)	50	The shape of segments basal leaves
16	Calyx length/ Calyx width (mm)	34	Bract width (mm)	51	Stamen filament color
17	Petal length (mm)	35	Bract length / Bract width (mm)	52	Stigma hair
18	Petal width (mm)	-	-	-	-

Table 3. Primer sequences were used in this study.

Region	Forward (5′→3′)	Reverse (5′→3′)	Reference
trnL5′-3′-trnF	CGAAATCGGTAGACGCTACG	ATTTGAACTGGTGACACGAG	Taberlet *et al*. (1991).
ITS	GGA AGT AAA AGTCGT AAC AAG G	TCCTCCGCTTATTGATATGC	White *et al*. (1990).

Molecular analyses

Relationship of species with ITS sequence

The relationship of species was investigated using various phylogenetic approaches including Bayesian statistics, maximum parsimony (MP), and maximum likelihood (ML). PAUP* program was used to conduct the maximum parsimony (MP) (Swofford, 2002). Each of the two single-region datasets used the heuristic search technique, based on tree bisection–reconnection (TBR) branch swapping, with 1000 replicas of random addition sequence. In the analysis, characters that were non-informative were excluded. A full heuristic search with 1000 bootstrap replicas (Felsenstein, 1985), each having a simple addition sequence, was used to calculate the values of branch support. To assess the combination ability of these two datasets, the partition-homogeneity test (incongruence length difference (ILD) test) proposed by Farris *et al.* (1995) was used in PAUP (Swofford, 2002). To conduct the test, invariant characters were used, the exclusion of which (Cunningham, 1997) occurred using the heuristic search technique including 100 replicas of the random addition sequence and TBR branch swapping with 1,000 homogeneity replicas. The maximum tree number was considered 500. MrModeltest software v. 2.3 (Nylander, 2004) was employed for the selection of the sequence evolution model for each dataset, with implementation in MrMTgui (Nuin, 2005) according to Akaike information criterion (AIC) (Posada and Backley, 2004). The analysis of all the datasets was conducted as a single partition with the Kimura 2-parameters + G model by Bayesian inference (BI) with MrBayes program v. 3.12 (Ronquist and Huelsenbeck, 2003). To estimate posteriors on the parameters of the model based on the data, the default priors were used. To perform this analysis, the Markov chain Monte Carlo method was used with four million generations. MrBayes was used to conduct two analyses at the same time, which started from various random trees (Nruns= 2), each with four Markov chain trees sampled every 100 generations. Of the trees, the first 25% were cast aside as burn-in while the rest were subsequently utilized for building a 50% threshold Majority-rule consensus tree together with values of posterior probability (PP). Tree View v. 1.6.6 was employed for visualizing trees.

Results

Morphometry

Species delimitation and inter-relationship

PCOA plot was drawn based on all the collected samples separated from plants of different species in separate groups or clusters (Fig. 2). Therefore, *Lonicera* species indigenous to Iran can be differentiated based on the studied morphological characters. Morphological characters used also could delineate the presumed species.

Based on the PCA analysis, more than 70% of the total variations belonged to the first three factors. The highest correlation (>0.7) of morphological characters in the first PCA axis having 48% of total variance belonged to the characters of stem hair, petiole hair, peduncle and pedicel hair, leaf hair, and petal width had.

In the WARD tree, two main clusters were created (Fig. not included), the first of which involved two subclusters: *L. nummulariifolia, L. caucasica*, and *L. korolkowii* plants comprised the first cluster. The other main cluster also had two subclusters: *Lonicera floribunda, L. iberica*, and *L. hypoleuca* had morphological similarities and thus sat adjacent to each other. Morphological characters used also could delineate the presumed species.

Molecular studies

ITS sequence-based phylogeny

An image of the ITS generated by the ITS4 primer is shown in Fig. 3. ITS dataset shows that the results of the maximum likelihood, maximum parsimony, and Bayesian approaches have close similarities. However, the manual comparison showed a higher degree of similarity between ITS and morphological characters' trees (Fig. 4).

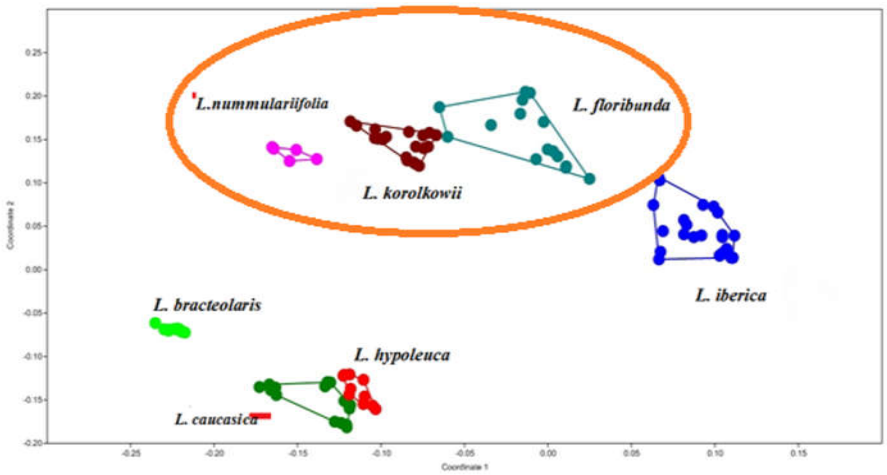

Fig. 2. PCOA plot of morphological characters revealing species delimitation in *Lonicera.*

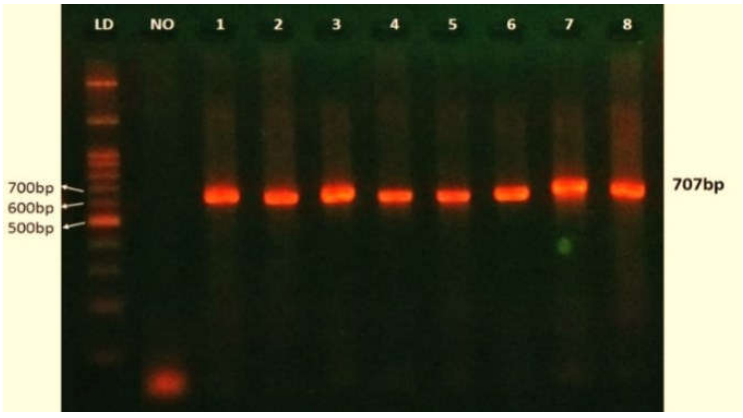

Fig. 3. Results of amplification with primer ITS on agarose 1.8% with 7 lanes gel tray. 1-8 individuals of *Lonicera.*

Here, Bayesian trees together with posterior probability (PP) and bootstrap based on ITS are only shown. In both ITS and Morphological characters' trees, the species *L. nummulariifolia, L. caucasica,* and *L. korolkowii* show close affinity, and similarly, species *Lonicera floribunda, L. iberica,* and *L. hypoleuca* are closely related.

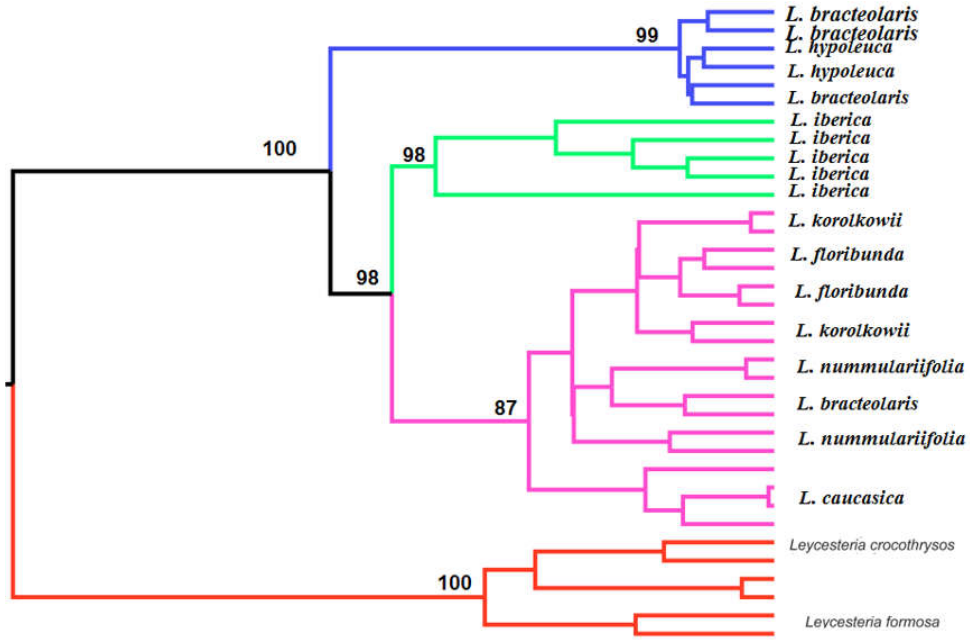

Fig. 4. Bayesian tree for the species phylogeny for seven *Lonicera* species and Two species of *Leycesteria* (*L. formosa* wall. and *L. crocothyrsos* Airy Shaw) were selected as outgroups, inferred by joint analysis of nrDNA ITS data, Branch support values are given as bootstrap (BP) value above branches.

Discussion

Recent years have seen significant progress in plant molecular and molecular phylogenetic research, which has led to dramatic changes in preconceived attitudes toward relationships among organisms and evolution at all taxonomic levels in the tree of life, ranging from the species and subspecies levels to kingdom and above-kingdom levels. This changed view about organismal relationships resulting from phylogenetic research is also transforming previous classification approaches in many plant groups. However, relying on one dataset can give rise to an improper answer or incorrect view of phylogenetic correlations. Therefore, using multiple datasets (both non-molecular and molecular preferably) for deriving phylogenetic information has become commonplace (Soltis and Soltis, 2000). However, despite the necessity of using several datasets to reliably estimate phylogenetic associations, various genes can have distinct histories, and thus, the phylogenetic trees they produce may not picture the true relationships, and different orthologous genes may usually give rise to tree topologies that are strongly supported but incompatible.

The three primary causes of incongruence in tree topologies are horizontal gene transfer, gene duplication, and deep coalescence; the importance levels of these causes are different according to the genes and taxa under study. Moreover, further sources of heterogeneity in gene trees are deep coalescence or incomplete lineage sorting, chloroplast capture, and branch length heterogeneity resulting from the coalescent process (Soltis and Soltis, 2000).

To handle several datasets in phylogenetic analysis, researchers have proposed three alternatives: *consensus*, *combined*, and *conditional combination* approaches. The conditional combination involves the combination of data except for cases in which there is considerable heterogeneity among datasets, which can be attributed to distinct branching histories (Soltis and Soltis, 2000). For this reason, many have suggested different statistical tests for phylogenetic trees congruence (see, for example, Foulds and Robinson, 1981). However, per several researchers, statistical congruence tests may

fail to give a decisive answer about the suitability of combining datasets. In other words, even in cases where congruence tests show a low heterogeneity level among datasets, combining datasets can be justified (Soltis and Soltis, 2000).

Currently, multispecies coalescent (MSC) approaches are regarded as novel approaches to estimate a species tree from a set of gene alignments. Based on new progress, MSC species phylogeny, gene phylogenies, and ancestral state reconstruction (ASR) of special characters understudy, like geographical of morphological evolution, can be estimated simultaneously (Bouckaert *et al.*, 2014).

We found morphological taxonomic identification was often congruent with nrDNA markers. The species relationship obtained in the ITS-based tree is also in agreement with the morphological tree.

Systematic and evolutionary aspects

PCoA plot of morphological characters separated each species; this is in agreement with phylogenetic analysis by using *ITS* sequences. This study documents the occurrence of 7 species belonging to the genus *Lonicera* that have been found in Iran. The most valuable characters in the genus in terms of taxonomy are the length of pedicel and bract and the width and length of the petal and stem leaves (Table 2). Four species and 12 populations of the genus *Lonicera* have been studied in terms of pollen and seed micro-morphology and molecular phylogeny (Amini *et al.*, 2019). Based on the findings, molecular and micro-morphological data present reliable evidence for the differentiation of some populations from others. Since *Lonicera* systematically is a problem genus, it is necessary to use alternative methods to distinguish its taxa. Statistical evaluation of taxa can be used for taxa delimitation. The present study intends to provide further evidence for taxonomists, to help them in separating these seven species. Our morphological results support close affinity between *L. iberica* and *L. hypoleuca,* as well as between *L. korolkowii* and *L. bracteolaris,* and these results are consistent with molecular findings. Our results correspond with the findings of Theis *et al.* (2008) and Nakaji *et al.* (2015).

As reported by Smolik *et al.* (2006), six *Lonicera periclymenum* populations have a similarity level that ranges from 82.3-86.6%; this indicates that they are closely related. Smolik *et al.* (2010) employed ISSR amplification for analyzing the microsatellite sequence polymorphism in the honeysuckle genome and evaluating genetic variety among 14 Russian and Polish blue honeysuckle accessions. Naugžemys *et al.* (2011) employed random amplified polymorphic DNA (RAPD) approach for assessing genetic associations among 51 blue honeysuckle accessions. The values of pairwise genetic distance (GDxy) varied in the 0.054-0.479 range among accessions under study, with a mean GDxy of 0.283. Knowing the contents of secondary metabolites in different genotypes provides the ability to choose the best in the breeding programs of *Lonicera* to increase health benefits and nutritional values.

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Conflict of interests

The authors declare that they have no conflicts of interest.

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