Micro-morphological and molecular study of four species of *Lonicera* (*Caprifoliaceae*) in Iran

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Abstract. *Lonicera* is one of the genera of *Caprifoliaceae* presented with nine species in Iran. In this study, the micro-morphological and molecular characters of 12 populations from four species of *Lonicera* (*L. bracteolaris, L. hypoleuca, L. iberica* and *L. korolkowii*) have been analyzed so as to evaluate their diagnostic value. Seven quantitative and qualitative characters of pollen were selected and measured. The most important characters include: shape, ornamentation of tectum, exine thickness, and P/E ratio of the pollen. On the basis of this study, the seed shape and surface contribute at least to differentiation of these species. Using nuclear (nrDNA ITS) markers, phylogenetic relationships within the four species of *Lonicera* have been reconstructed. Then the data set was analyzed by phylogenetic methods including Bayesian, Maximum Likelihood, and Maximum Parsimony methods. In phylogenetic analyses, all members of the four species formed a well-supported clade (PP=1, ML/BS=100/100) and divided into three major clades (I, II and III). The Neighbor Net Diagram supported the phylogenetic results. The results showed that micro-morphological and molecular data provide reliable evidence for differentiation of some populations from others.

Key words: Iran, *Lonicera*, micro-morphological, molecular, pollen, seed

Introduction

Lonicera L. (*Caprifoliaceae*) includes more than 180 species (Mabberley 2008) worldwide, with 19 species in the region of Flora Iranica (Wendelbo 1965). The genus is mainly distributed in temperate to subtropical regions of the northern hemisphere: Europe, Russia, East Asia, and North America (Hsu & Wang 1988; Mabberley 2008). In the flora of Iran, the genus *Lonicera* is represented by nine species (Khatamsaz 1995; Ghahremaninejad & Ezazi 2009) across the north, northwest and northeast of the country. Some species are medicinal plants (Zeng & al. 2017). Dried *Lonicera* flowers and buds are known as Flos Lonicera and have been a recognized herb in the traditional Chinese medicine for more than 1500 years (Li & al. 2015). It has been applied for treatment of arthritis, diabetes mellitus, fever, and viral infections (Shang & al. 2011; Li & al. 2015). The plants are erect shrubs, occasionally small trees. Members of *Lonicera* are characterized by opposite, narrowly elliptic to obovate leaves, white, yellow, reddish, or purple-red corolla with capitate stigma (Judd & al. 2007), and undulate calyx margin.

Historically, *Lonicera* has received the widest-scale taxonomic evaluation. Rehder (1903, 1913) divided *Lonicera* into two subgenera: *Lonicera* and

Caprifolium. *Caprifolium* is the smaller subgenus in the genus *Lonicera*. Morphologically, the subgenera are distinctive, most significantly by the inflorescence, the former with two-flowered cymes, and the latter with three-flowered cymes. Four sections are generally recognized in the subgenus *Lonicera*: *Coeloxylosteum* Rehder, *Isoxylosteum* Rehder, *Nintooa*, and *Isika* (Adams.) Rehder (Rehder 1903; Hara 1983; Hsu & Wang 1988). In *Flora Iranica,* Wendelbo (1965) classified 19 species of the *Lonicera* into two subgenera (*Chamaecerasus* and *Lonicera*) and three sections, namely *Isoxylosteum*, Isika and *Coeloxylosteum.* The four studied species belong to subgenus *Chamaecerasus* and sections *Isika* and *Coeloxylosteum*. Nakai (1938) assigned the Japanese species of *Lonicera* to 15 sections and eight subsections. Subsequently, Hara (1983) improved Nakai's system for the Japanese species. Following Rehder, Hsu & Wang (1988) proposed a new system for the Chinese species of *Lonicera*.

Micro-morphological characters have good diagnostic value in distinguishing many taxa, principally at the species level. Palynological studies for this genus are limited (Grigoryevaet & al. 2014; Perveen & Qaiser 2007). Pollen morphology of 18 species of the family *Caprifoliaceae* was investigated by Perveen & Qaiser (2007) from Pakistan. On the basis of the exine pattern, six distinct pollen types have been recognized: *Abeliatriflora*-type, *Lonicera myrtillus*-type, *Lonicera obovata*-type, *Lonicera quinquelocularis*, *Lonicera webbiana*-type, and *Viburnum grandiflorum*. Grigoryeva & al. (2014) has studied 22 species of *Lonicera* and has found that the pollen grains of *Lonicera* are large, 3-4(5-6)-colporate, subspheroidal, with echinate exine. Jacobs & al. (2009) studied the evolution of fruit and seed characters in the *Diervilla* and *Lonicera* clades. They showed that the seeds of *Lonicera* are dorsiventrally compressed and irregular in shape.

Molecular data have been obtained in phylogenetic studies and species divergence researches (Kazempour Osaloo & al. 2003, 2005). These data can also provide supportive and extra criteria for systematic classification of the studied species that have been based only on the morphological characters (Chase & al. 1993). The internal transcribed spacer (ITS) is the region of the 18S-5.8 S-26S nuclear ribosomal cistron (Baldwin & al. 1995). The spacers contain the signals needed to process the rRNA transcript (Baldwin 1992, Baldwin & al. 1995) and have often been used for inferring phylogeny at the generic and infrageneric levels in plants (e.g. Baldwin 1992; Baldwin & al. 1995; Kazempour Osaloo & al. 2003, 2005; Ahangarian & al. 2007). Theis & al. (2008) studied phylogenetics of the *Caprifolieae* and *Lonicera* (*Dipsacales*) on the basis of nuclear and chloroplast DNA sequences. Their analysis indicates monophyly in *Lonicera* and highlights instances of homoplasy in several morphological characters. Molecular phylogenetics of *Lonicera* L. (*Caprifoliaceae*) in Japan has been studied by Nakaji & al. (2015) on the basis of chloroplast DNA sequences. According to the results, circumscription of the higher taxonomic groups for the Japanese species of *Lonicera* proposed by Hara in 1983 is fundamentally acceptable.

Lonicera is well known for its taxonomic complexity resulting from overlapping morphological characters. There is no comprehensive systematic study of *Lonicera* species in Iran. This research presents the first comprehensive issue on the systematic significance of pollen and seed characters in the Iranian species of *Lonicera.* Thus, the objectives of present study are: (1) to find diagnostic micro-morphological characters for distinguishing the closely related species; (2) to use the pollen grains and seed features as a source of diagnostic characters in these species; (3) to investigate the molecular properties of *Lonicera* in Iran; (4) to evaluate the affinities and relationships of its four species.

Material and methods

In the present study, 12 populations from four species of *Lonicera* (*L. bracteolaris* Boiss. & Buhse, *L. hypoleuca* Decne., *L. iberica* M. Bieb. and *L. korolkowii* Stapf) were obtained from almost every region in northern Iran during fieldwork from the beginning of March 2016, and to the end of July 2016 (Table 1).

Some of the collected specimens were dried according to standard procedures and stored as herbarium specimens for use in morphological investigations. The above-mentioned plants were kept in the Gonbad Kavous University Herbarium (GKUH). *Flora Iranica* (Wendelbo 1965) was used for identification.

Morphological methods

Palynological studies were carried out with a light microscope (LM) and scanning electron microscope (SEM) on pollen grains of *L. bracteolaris*, *L. hypoleuca*,

Table 1. List of species used in the study, along with localities and vouchers.

Taxa	Collection data (all samples are from Iran)	GenBank accession no. ITS
L. bracteolaris Boiss. & Buhse	Golestan: Gorgan, Tuskestan forest, Khormali &Sattarian, GKUH	LC466560
L. bracteolaris	Golestan: Chino, Khormali&Sattarian, GKUH	LC466561
L. bracteolaris	Golestan: Tilabad, Khormali&Sattarian, GKUH	LC466562
L. hypoleuca Decne.	Golestan: Golestan National Park, Khormali&Sattarian, GKUH	LC466563
L. hypoleuca	Khorasan: North khorasan, Khormali&Sattarian, GKUH	LC466564
L. hypoleuca	Khorasan: North khorasan, Khormali&Sattarian, GKUH	LC466565
L. iberica M.Bieb.	Golestan: Kordkuy, Khormali&Sattarian, GKUH	LC466566
L. iberica	Golestan: Deland, Khormali&Sattarian, GKUH	LC466567
L. iberica	Golestan: Gorgan, Naharkhoran, Khormali&Sattarian, GKUH	LC466568
L. korolkowii Stapf	Golestan: Golestan forest, Khormali&Sattarian, GKUH	LC466569
L. korolkowii	Golestan: Bandar-e Torkman, Khormali&Sattarian, GKUH	LC466570
L. korolkowii	Golestan: Bandar-e Gaz, Khormali&Sattarian, GKUH	LC466571

L. iberica, and *L. korolkowii*. The pollen samples were obtained mostly from freshly collected herbarium specimens. For LM studies, the samples were acetolyzed following Erdtman's technique (Erdtman 1952). The measurements were based on at least 30 pollen grains per population performed with the help of a Nickon light microscope and a Canon digital camera.

For SEM investigations, the pollen grains were transferred directly to double-sided tape-affixed stubs and were sputter-coated with gold. Photomicrographs were taken with a VEGA//TESCAN-LMU electron microscope at an accelerating voltage of 15–22 kV at the Research Institute of Razi, Tehran, Iran. The applied terminology is based on Punt & al. (2007).

Seeds of the four species of *Lonicera* (*L. bracteolaris*, *L. hypoleuca*, *L. iberica* and *L. korolkowii*) were taken from herbarium specimens. The samples of every species were examined under the stereomicroscope to ensure the normal size and maturity of the specimen mounted directly on aluminum stubs with the help of two-sided adhesive tape. After coating with a thin layer (*ca*. 25 nm) of gold, they were analyzed under VEGA// TESCAN-LMU electron microscope, at an accelerating voltage of 15–22 KV at the Research Institute of Razi, Tehran, Iran. At least 10 seeds were assessed by biometric methods in order to record the morphological and size parameters, seed type, ornamentation character, and color status. The list of voucher specimens and details of localities is given in Table 1.

In order to detect significant differences in the studied characters of the various studied species, an analysis of variance (ANOVA) was performed. To reveal the species relationships, cluster analysis and principal component analysis (PCA) (Ingrouille 1986) were applied. The average taxonomic distances and squared Euclidean distances were used as dissimilarity coefficient in the cluster analysis of morphological data. In order to determine the most variable pollen characters among the studied species, factor analysis based on principal components analysis was performed by SPSS ver. 19 (2010).

Molecular methods

Taxon sampling. Four species of *Lonicera* and 12 populations were chosen as in-group for nrDNA ITS[1]. Two species of *Leycesteria* (*L. formosa* wall. and *L. crocothyrsos* Airy Shaw) were selected as outgroups in line with the earlier molecular phylogenetic studies (Theis & al. 2008; Jacobs & al. 2009; Nakaji & al. 2015). A list of all taxa used in this study, as well as the sources, voucher information and GenBank accession numbers are given in Table 1.

DNA extraction, PCR and sequencing. Using the Kit Method, total genomic DNA was extracted from dried leaf material deposited in the Gonbad Kavous University Herbarium (GKUH). The nrDNA ITS region was amplified with primers ITS5m of Sang & al. (1995) and ITS4 of White & al. (1990). PCR amplification of the DNA regions followed procedures described in detail by Naderi Safar & al. (2014). The quality of PCR products was checked by electrophoresis in 1 % agarose gel in 1 × TAE (pH = 8) buffer and they were photographed with a UV gel documentation system (UVItec, Cambridge, UK). Along with the primers, PCR products were sent for Sanger sequencing at Macrogen (Seoul, South Korea) via Pishgam Inc., Tehran-Iran.

[1] nuclear ribosomal DNA internal transcribed spacer

Sequence alignment. Each single dataset was aligned using the web-based version of MUSCLE (Edgar 2004; at http://www.ebi.ac.uk/Tools/msa/muscle/) under default parameters, followed by manual adjustment. The alignment of datasets required numerous single- and multiple-base indels (insertions/deletions). Position of indels was treated as missing data for all datasets.

Phylogenetic inferences

Parsimony method. Maximum parsimony (MP) analyses were conducted using PAUP* version 4.0a157 (Swofford 2002). A heuristic search option was employed for each dataset with tree bisection-reconnection (TBR) branch swapping, 1000 replications of random addition sequence and automatic increase in the maximum number of trees. Uninformative characters were excluded from the analyses. Branch support values (MPBS) were estimated by full heuristic search with 1000 bootstrap replicates (Felsenstein 1985), each with a simple addition sequence.

Likelihood method. Maximum likelihood analysis (ML) was performed on each dataset with RAxML Ver. 8.2.10 (Stamatakis 2014), as implemented in the CIPRES Science Gateway (Cyber Infrastructure for Phylogenetic Research Cluster) (Miller & al. 2010, https://www.phylo.org). The evolution model employed for each dataset was the same as that of Bayesian analyses. Bootstrap values (MLBS) were calculated in RAxML, based on 1000 replicates with one search replicate per bootstrap replicate. Generally, mean p-distance for each dataset was computed using MEGA7 (Kumar & *al.* 2016).

Bayesian inference. For Bayesian inference (BI) analyses, models of sequence evolution were selected with the program Mr Modeltest, version 2.3 (Nylander 2004) based on the Akaike information criterion (AIC) (Posada & Buckley 2004). This program indicated a GTR+G model for nrDNA ITS as the best model for nucleotide substitution. BI analysis was performed using Mr Bayes version 3.2 (Ronquist & al. 2012) on the CIPRES Science Gateway for the datasets. Bayesian analyses were performed, with default priors (uniform priors) and the best-fit model of sequence evolution for each dataset, with two runs of ten million generations and four simultaneous chains (one cold and three heated, with a heating parameter of 0.2), by saving trees every 100 generations. The trees, sampled after discarding 25 % as "burn-ins", were collected to build a 50 % majority rule consensus phylogram to calculate posterior probability values (PP). Tree visualization was effected by using Tree View version 1.6.6 (Page 2001).

Phylogenetic networks. Neighbor Net (NN), a distance-based network construction method (Bryant & Moulton 2004), was used in SPLITS TREE4, version 4.14.4 (Huson 1998), applying a Dice dissimilarity matrix. The ITS matrix was modified prior to analysis by excluding the outgroups.

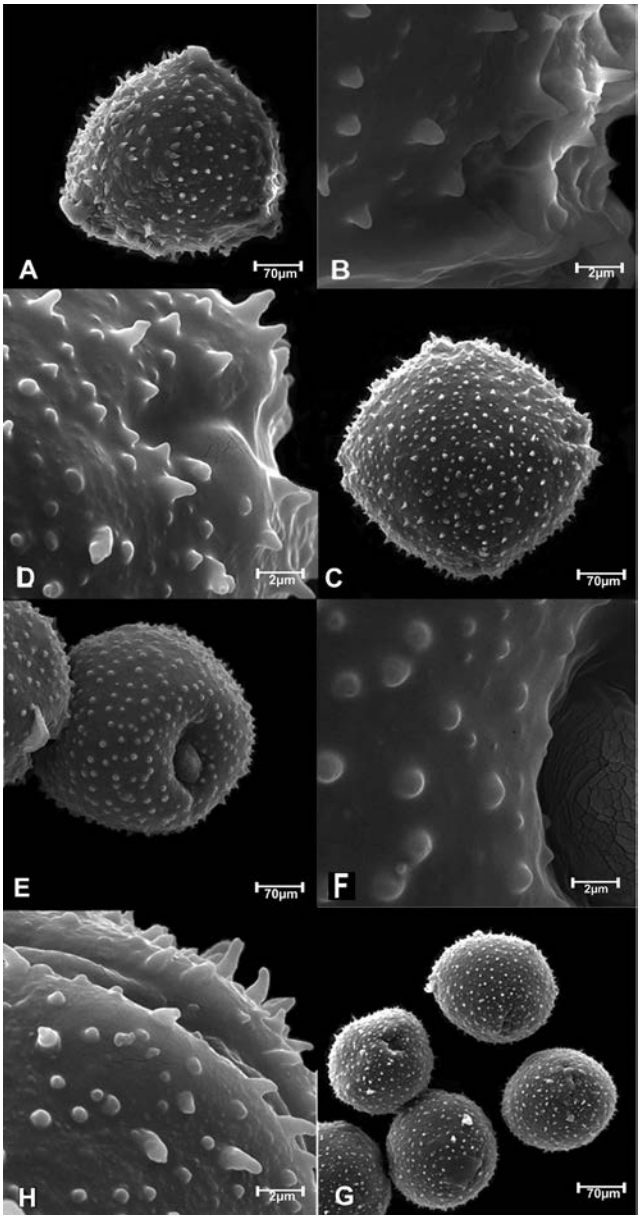
Results

Pollen morphology

The pollen grains of the studied species revealed variations and distinguished four species of *Lonicera*. All palynological structures and measurements of the examined species concerning the pollen type – polar view, polar (P) and equatorial (E) measurements, P/E ratio, pollen shape, and tectum ornamentation are shown in Table 2. Selected SEM micrographs of the pollen grains and their surfaces are shown in Fig. 1. Generally, polar and equatorial axis were regarded as useful in separating the four species. Polar axis (P) length of the pollen grains ranged from the smallest for *L. iberica* (44.65μm) to the greatest for *L. korolkowii* (67.45 μm). Equatorial axis (E) length of the pollen grains ranged from the smallest in *L. iberica* (49.43μm) to the greatest in *L. korolkowii* (72.05 μm). The shape classes were based on the ratio between the length of polar axis (P) and equatorial diameter (E). The P/E ratio ranged from 0.86 μm to 0.93 μm; therefore, the pollen grains were triangular to quadrangular, or circular and prolate spheroidal. The smallest and largest exine thickness was observed in *L. iberica* (2.34 μm) and *L. korolkowii* (3.78), respectively. Tectum ornamentation was spinulose in *L. bracteolaris* (Fig. 1B), microechinate-granulate in *L. hypoleuca* (Fig. 1D), or granulate in *L. iberica* (Fig. 1F) and echinate in *L. korolkowii* (Fig. 1H). In order to define the diagnostic value of pollen grains in the species delimitation in studied *Lonicera* species, cluster analysis by Ward's method was performed on the basis of seven qualitative and quantitative characters (Fig. 2). Ward's dendrogram showed two main clusters (Fig. 2). The first cluster was composed of *L. iberica* and *L. hypoleuca*. The second cluster was composed of two subsets and contained *L. bracteolaris* and *L. korolkowii,* plus two populations of *L. iberica* (Fig. 2). Factor analysis revealed that there were two factors, which provided more than 78 % of all observed variations in the studied

Table 2. Pollen morphological characters of the examined taxa of *Lonicera*.

Taxa	Polar axis (µm)	Equatorial axis (µm)	P/E (µm)	Shape	Colpus length (µm)	Exin thickness (µm)	Tectum
L. bracteolaris Boiss. & Buhse	59.43±0.11	67.77±0.43	0.88	Triangular	16.60±0.12	3.23±0.05	Spinulate
L. bracteolaris	60.05±0.17	65.65±0.27	0.92	Triangular	16.80±0.09	3.14±0.01	Spinulate
L. bracteolaris	58.75±0.22	67.37±0.32	0.86	Triangular	16.70±0.15	3.05±0.09	Spinulate
L. hypoleuca Decne.	48.65±0.19	54.43±0.35	0.88	Quadrangular	15.16±0.16	2.74±0.04	Microechinate - Granulate
L. hypoleuca	49.79±0.18	55.67±0.29	0.89	Quadrangular	15.96±0.07	2.63±0.01	Microechinate - Granulate
L. hypoleuca	48.73±0.31	54.35±0.34	0.88	Quadrangular	15.36±0.21	2.85±0.03	Microechinate - Granulate
L. iberica M.Bieb.	44.65±0.36	49.43±0.31	0.89	Circular	15.10±0.16	2.70±0.01	Granulate
L. iberica	46.75±0.41	51.47±0.44	0.90	Circular	14.98±0.12	2.34±0.07	Granulate
L. iberica	47.64±0.34	53.35±0.17	0.88	Circular	15.49±0.28	2.45±0.04	Granulate
L. korolkowii Stapf	65.44±0.38	70.65±0.28	0.92	Prolate spheroidal	17.70±0.19	3.78±0.06	Echinate
L. korolkowii	64.30±0.29	71.25±0.26	0.90	Prolate spheroidal	17.66±0.30	3.54±0.08	Echinate
L. korolkowii	67.45±0.42	72.05±0.19	0.93	Prolate spheroidal	17.53±0.32	3.65±0.06	Echinate



pollen grains. Study of the component matrix for each factor showed that shape and ornamentation of tectum were the most important traits for the first factor; exin thickness and P/E ratio were most significant for the second factor. PCO confirmed the results of cluster analysis by Ward's method based on the qualitative and quantitative characters of pollen grains (Fig. 3).

Seed characteristics

Values of six quantitative and qualitative seed traits have been observed and measured in the four *Lonicera* species given in Table 3. SEM photographs for each species, showing the seed character variations, are given in Fig. 4. Seeds were generally almond-shaped, with various degrees of deviation. However, circular almond-shaped seeds were also observed occasionally among some of the examined species. The greatest length of the seeds – 3.48 mm was observed in *L. korolkowii* (column 2 in Table 3), and smallest width – 1.07 mm was registered in *L. iberica* (column 3, Table 3). The length/width ratio varied between 1.14 mm in *L. hypoleuca* to 1.91 mm in *L. iberica*. In terms of exomorphology, seed surface was generally irregularly papillose in *L. bracteolaris* (Fig. 4B), rounded in *L. hypoleuca* (Fig. 4D), polygonal in *L. iberica* (Fig. 4F), and elongated in *L. korolkowii* (Fig. 4H). The anticlinal walls were shallow in *L. bracteolaris*, deep in *L. hypoleuca*, very deep in *L. iberica,* and indistinct in *L. korolkowii* (Figs. 4B, D, F, H).

Fig. 1. Scanning electron micrographs (SEM) of pollen surface in *L. bracteolaris*, *L. hypoleuca, L. iberica,* and *L. korolkowii*. For each taxon, the first micrograph shows the outline of the pollen grain indicating its general shape, and the second micrograph is a close view of the pollen surface. (A1, A2) *L. bracteolaris*, (B1, B2) *L. hypoleuca*, (C1, C2) *L. iberica*, (D1, D2) *L. korolkowii*.

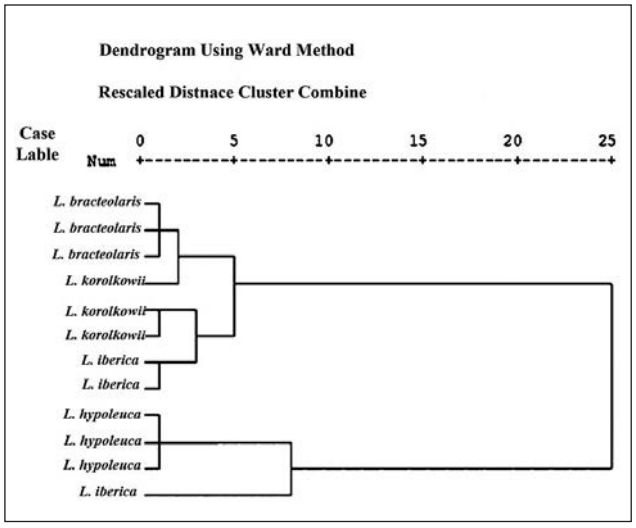


Fig. 2. Cluster analysis (Ward's method) based on pollen features of *Lonicera*.

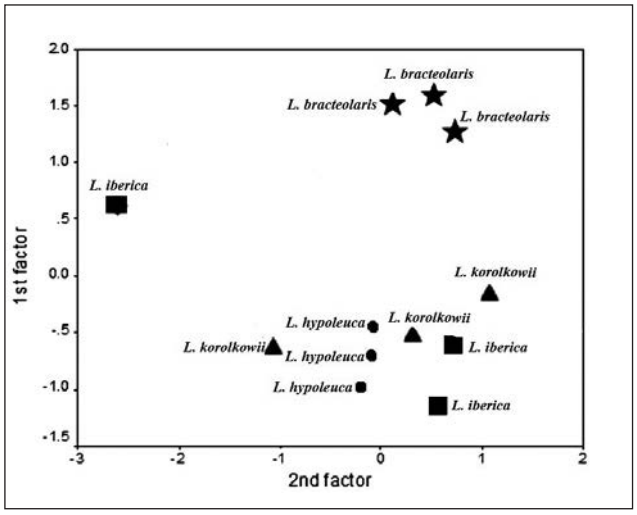


Fig. 3. PCO plot of *Lonicera* species based on the observed pollen data.

Phylogenetic analysis

Detailed information about alignment characteristics, selected model of nucleotide substitution, as well as tree statistics from the single analysis of the nrDNA ITS region are summarized in Table 4. The aligned nrDNA ITS matrix comprised 698 characters. The parsimony and Bayesian analyses of the nrDNA ITS produced congruent trees and gave similar results. All members of this genus formed a well-supported clade (PP =1, ML/BS =100/100) and three major groups were detected (Fig. 5). Clade I is composed of *L. iberica* and *L. hypoleuca.* Two populations of *L. korolkowii* were nested in Clade II, while other members of *L. korolkowii* and *L. bracteolaris* were placed in Clade III.

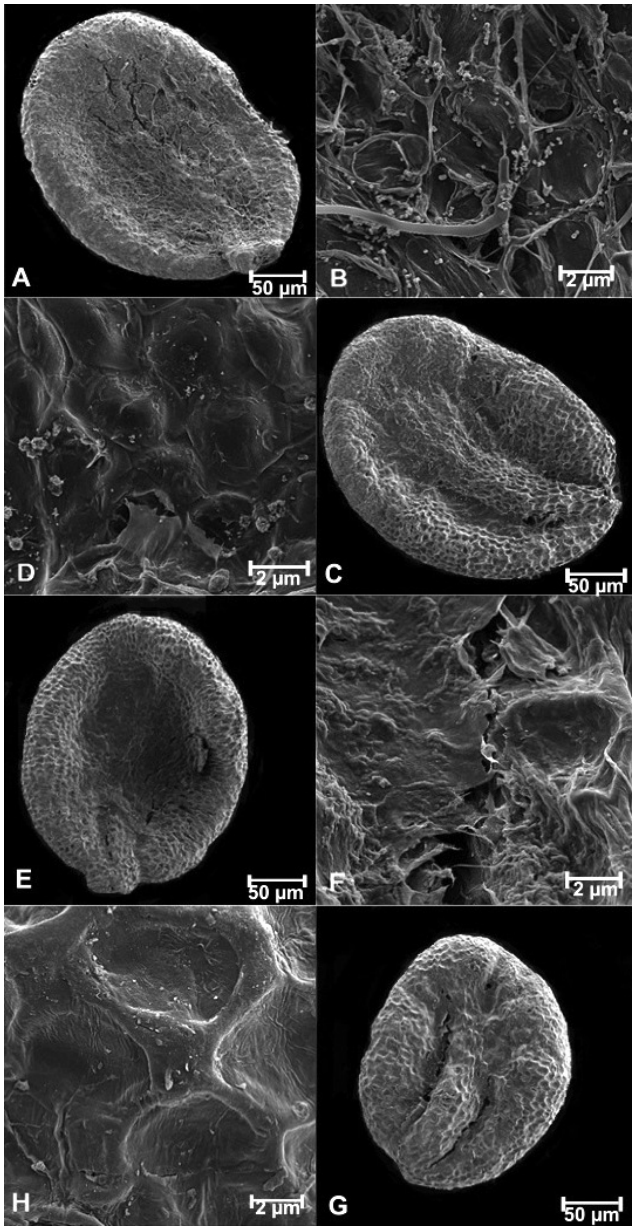


Fig. 4. Scanning electron micrographs (SEM) of the seed surface in *L. bracteolaris, L. hypoleuca, L. iberica*, and *L. korolkowii*. For each taxon, the first micrograph shows the outline of the seed indicating its general shape, and the second micrograph is a close view of the seed surface. (A1, A2) *L. bracteolaris,* (B1, B2) *L. hypoleuca,* (C1, C2) *L. iberica,* (D1, D2) *L. korolkowii*.

Phylogenetic networks

The splits graph showed extensive internal network structure, indicating reticulation. Correlation between geographical and genetic distance of the studied populations (Podani 2000) was checked. The groups formed in the splits graph were readily correlated to the clades recovered in the phylogenies. Populations of *L. iberica* and *L. hypoleuca* (1, 2, 3, 4, 5, and 6) were distinct and stood out at major distance

separately from the other populations (Fig. 6). The populations of *L. korolkowii* and *L. bracteolaris* (7, 8, 9, 10, 11, and 12) showed a closer genetic affinity and were placed close to each other (Fig. 6).

Discussion

Lonicera is one of the most important genera of *Caprifoliaceae*. Four species and 12 populations of the genus *Lonicera* have been studied in terms of pollen and seed micro-morphology and molecular phylogeny. *Lonicera* has attracted little attention in earlier micromorphological and phylogenetic studies, hence, this study presents the first comprehensive investigation of this genus in Iran. Micro-morphological evaluation of the *Lonicera* species has shown the diagnostic value of these characters. Analysis has shown that *L. korolkowii* has the largest, while *L. iberica* has the smallest pollen grains (Table 2). The present study shows that *Lonicera* pollen grains are triangular to quadrangular, or circular and prolate spheroidal, while Perveen & Qaiser (2007) have mentioned the shape of the Pakistani species as oblate-spheroidal, seldom sub-oblate. Although Grigoryeva & al. (2014) believe that there is no significant difference between the *Lonicera* species in terms of pollen grains, the species in the present study have manifested clearly distinct elements by means of a selected set of characters. Our palynological investigations of different *Lonicera* species have confirmed the importance of pollen characters for taxa delimitation. As it is evident from Ward's dendrogram, the species are not distinguished solely by their pollen characters and pollen morphology seems insufficient for their identification. The results have shown that pollen morphology provides reliable evidence for delimitation of some populations from the others.

The present study emphasizes the findings of Jacobs & al. (2009) that seed shape in the *Lonicera* species is dorsiventrally compressed and irregular. The seed surface distinguishes clearly the species. Another diagnostic character is the seed shape, thus *L. bracteolaris* and *L. korolkowii* are almond-shaped, *L. iberica* and *L. hypoleuca* are almond-shaped to circular. Analysis has shown that *L. korolkowii* has the largest, while *L. iberica* has the smallest seeds. Singularly, some seeds have hairs like in the 5ornamentation of *L. bracteolaris*.

Recent years have witnessed an enormous advance in the plant molecular studies and molecular phylogenetic investigations have dramatically reshaped the views on organismal relationships and

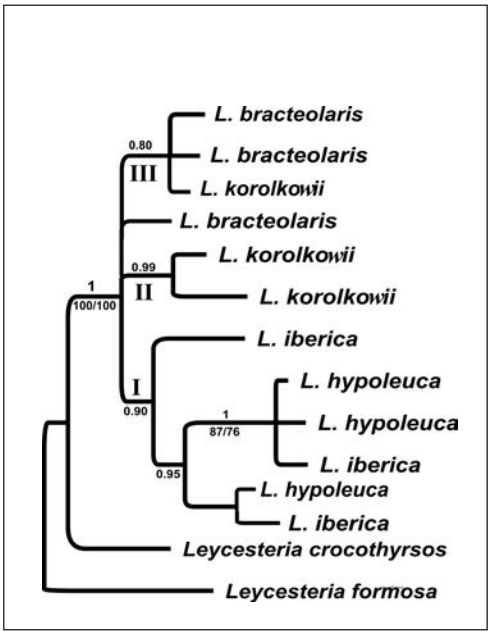


Fig. 5. Fifty percent majority-rule consensus tree resulting from the Bayesian phylogenetic analysis of the nrDNA ITS dataset. Numbers above and below the branches are posterior probability (PP) from the BI and bootstrap support (BS) values from a MP analysis, respectively. Values <50 % were not shown.

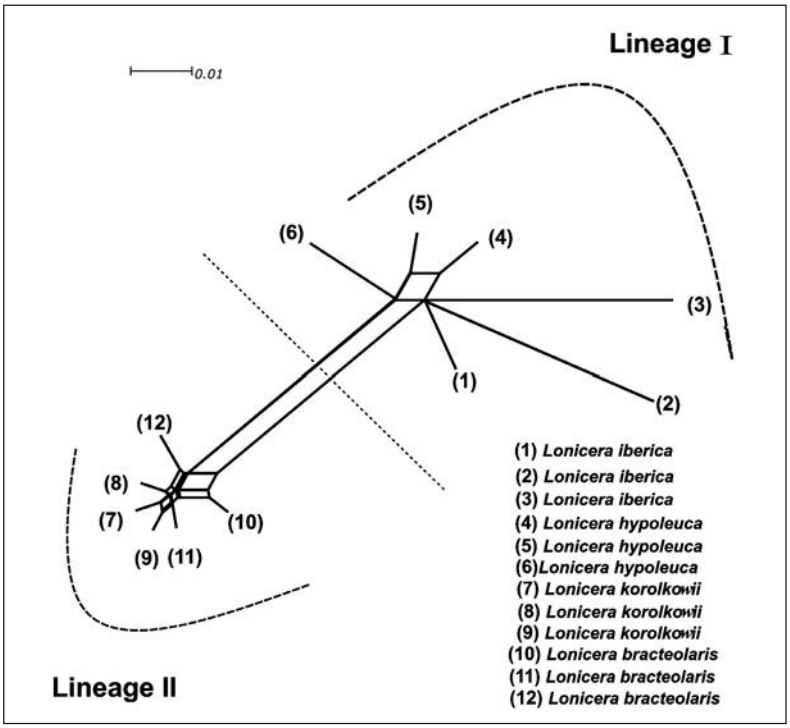


Fig. 6. Splits graph for ITS sequences of *Lonicera*. Two major groups are recovered (i.e., lineage I and lineage II).

evolution at all taxonomic levels of life hierarchy: from the species level (and below) to kingdom level (and above) (Soltis & Soltis 2000). Nuclear molecular technique has been successfully used for investigation of infraspecific variations in different genera (Sheidai & al. 2013, 2014; Koohdar & al. 2016). Therefore, this study uses molecular approach for investigation of infraspecific variations between the *Lonicera* species. Phylogenetic analysis has displayed monophyly in four *Lonicera* species, with strongly supported (PP=1, ML/BS=100/100) and resolved relationships between the species. Whereas monophyly of all four species is well sustained, interspcific relationships are less clear (Fig. 5). Our molecular 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 micromorphological findings regarding the characters of pollen and seeds.

Our results correspond with the findings of Theis & al. (2008) and Nakaji & al. (2015). All currently studied species are monophyletic and divided into three major well-supported clades. Clade I is composed of *L. iberica* and *L. hypoleuca*. Two populations of *L. korolkowii* are nested in Clade II, while other members of *L. korolkowii* and *L. bracteolaris* are placed in Clade III. This is probably due to hybridization among the *Lonicera* species, as Theis & al. (2008) have already mentioned. Hybridization may be the cause of phylogenetic incongruence among the species.

The Neighbor Net diagram (Fig. 6) has revealed some of the studied populations as separate within the network, supporting the phylogenetic results. The splits graph has shown extensive internal network structure indicating reticulation. The groups formed in the splits graph are readily correlated (with minor exceptions) to the clades recovered in the phylogenies, especially those with good support. The term "lineage" is used in reference to groups of specimens in the NN trees (Fig. 6), and "clade" in reference to groups in the phylogenies (Fig. 5). The ITS splits graph has revealed two main groups (Fig. 6). One of these, lineage "I" correlates to clade "I" in Fig. 5 and is composed of the populations of *L. iberica* and *L. hypoleuca*. The latter, lineage "II", includes the populations of *L. korolkowii* and *L. bracteolaris* corresponding to clade "II" and "III" in Fig. 5.

Conclusions

Apparently, reliance on a single data set may result in indistinct resolution or an erroneous picture of phylogenetic relationships. Moreover, it is necessary to use chloroplast markers to distinguish better the relationships and it is desirable to examine further the evolutionary history of the genus, with extensive taxon sampling. 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, so as to help them in separating these four species.

References

Ahangarian, S., Kazempour Osaloo, S. & Maassoumi, A. A. 2007. Molecular phylogeny of the tribe Hedysareae with special reference to *Onobrychis* (Fabaceae) as inferred from nrDNA ITS sequences. – Iran. J. Bot., **13**: 64-74.

Baldwin, B.G., Sanderson, M.J., Porter J.M., Wojciechowski, M.F., Campbell, C.S.& Donoghue, M.J. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. – Ann. Missouri Bot. Gard., **82**: 247-277.

Baldwin, B.G. 1992. Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: An example from the *Compositae*. – Mol. Phylog. Evol., **1**: 3-16.

Bryant, D. & Moulton, V. 2004. Neighbor-Net: An agglomerative method for the construction of phylogenetic networks. – Molec. Biol. Evol., **21**: 255-265.

Chase, M.W., Soltis, D.E., Olmstead, R.G., & 39 others. 1993. Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene rbcL. – Ann. Missouri Bot. Gard., **80**: 528-580.

Edgar, R.C. 2004. Muscle: Multiple sequence alignment with high accuracy and high throughput. – Nucleic Acids Res., **32**: 1792-1797.

Erdtman, G. 1952. Pollen Morphology and Plant Taxonomy. Angiosperms. ChronicaBotanica Co., Waltham, Massachusetts. Copenhagen.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. – Evolution, **39**: 783-791.

Ghahremaninejad, F., Ezazi, A. 2009. A new record for the flora of Iran: *Lonicera microphylla* (*Caprifoliaceae*). – Iran. J. Bot., **15** (2): 157-159.

Grigoryeva, V.V., Britski, D.A., Byalt, A.V. 2014. Pollen morphology of some species of *Lonicera* (*caprifoliaceae*) from Northwestern Russia. – Bot. Zhurn., **15**: 529-539.

Hara, H. 1983. A revision of *Caprifoliaceae* of Japan with reference to allied plants in other districts and the *Adoxaceae*. – Ginkgoana, **5**: 136-173.

Hsu, P.S. & Wang, H.J. 1988. *Lonicera* Linn. – In: Flora Republicae Popularis Sinicae. Beijing, **72**: 143-259.

Huson, D.H. 1998. Splits Tree: A program for analyzing and visualizing evolutionary data. – Bioinformatics, **14**: 68-73.

Ingrouille M. J. 1986. The construction of cluster webs in numerical taxonomic investigations. – Taxon, **35**: 541-545.

Jacobs, B. Lens, F. Smets, E. 2009. Evolution of fruit and seed characters in the *Dievilla* and *Lonicera* clades (*Caprifoliaceae*, *Dipsacales*). – Ann. Bot., **104**: 253-276.

Judd, W.S., Campbell, C.S., Kellogg, E.A., Stevens, P.F. & Donoghue, M.J. 2007. Plant Systematics: A Phylogenetic Approach, 3rd ed. Sunderland, MA: Sinauer.

Kazempour Osaloo, S., Maassoumi, A.A. & Murakami, N. 2003. Molecular systematics of the genus *Astragalus* L. (*Fabaceae*): Phylogenetic analyses of nuclear ribosomal DNA internal transcribed spacers and chloroplast gene ndhF sequences. – Pl. Syst. Evol. **242**: 1-32.

Kazempour Osaloo, S., Maassoumi, A.A. & Murakami, N. 2005. Molecular systematics of the Old World *Astragalus* (*Fabaceae*) as inferred from nrDNA ITS sequence data. – Brittonia, **57**: 367-381.

Khatamsaz, M. 1995. *Caprifoliaceae*. – In: Assadi, **M. & al.** (eds), Flora of Iran, no. 13. Tehran.

Koohdar, F., Sheidai, M., Talebi, S.M., Noormohammadi, Z. & Ghasemzadeh-Baraki, S. 2016. Genetic diversity, population structure and morphological variability in the *Lallemantiaroyleana* (*Lamiaceae*) from Iran. – Phytol. Balcan., **22**: 29-38.

Kumar, S., Stecher, G. & Tamura, K. 2016. MEGA7: molecular evolutionary genetics analysis, ver. 7.0 for bigger datasets. – Mol. Biol. Evol., **33**: 1870-1874.

Li, Y., Cai, W., Weng, X., Li, Q. Wang, Y., Chen, Y.W., Zhang, Q., Yang, Y., Guo, X., & Wang, H. 2015. Lonicerae japonica flos and lonicera flos: A systematic pharmacology review. Evid. Based Complement. Vol. 2015. – Alternat. Med., 905063.

Mabberley, D.J. 2008. The Plant Book, a Portable Dictionary of Higher Plants. Cambridge Univ. Press, Cambridge.

Miller, M.A., Pfeiffer, W. & Schwartz, T. 2010. Creating the CIPRES science gateway for inference of large phylogenetic trees. – Proc. Gateway Computing Environments Workshop (GCE), New Orleans, Louisiana. Piscataway: IEEE, 45-52.

Naderi Safar, K., Kazempour Osaloo, S., Maassoumi, A.A. & Zarre, S. 2014. Molecular phylogeny of *Astragalus* section *Anthylloidei* (*Fabaceae*) inferred from nrDNA ITS and plastid *rpl*32-*trn*L$_{(UAG)}$ sequence data. – Turk. J. Bot., **38**: 637-652.

Nakai, T. 1938. A new classification of the genus *Lonicera* in the Japanese empire, together with the diagnoses of new species and new varieties. – J. Jap. Bot., **14**: 359-376.

Nakaji, M., Tanaka, N. & Sugawara, T.2015. A molecular phylogenetic study of *Lonicera* L. (*Caprifoliaceae*) in Japan based on chloroplast DNA sequences. – Acta Phytotax. Geobot., **66** (3): 137-151.

Nylander, J.A.A. 2004. Mr Modeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala.

Page, D.M. 2001. Tree view (Win32) version 1.6.6. Available: http://taxonomy.zoology.gla.ac.uk/rod/treeview.html.

Perveen, A. & Qaiser, M. 2007. Pollen flora of Pakistan – lv. *Caprifoliaceae*. – Pakistan J. Bot., **39**(5): 1393-1401.

Podani, J. 2000. Introduction to the Exploration of Multivariate Data. Backhuyes, Leiden, **407** pp.

Posada, D. & Buckley, T.R. 2004. Model selection and model averaging in phylogenetics: Advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. – Syst. Biol., **53**: 793-808.

Punt, W., Hoen, P.P., Blackmore, S., Nilsson, S. & Thomas, A.L. 2007. Glossary of pollen and spore terminology. – Rev. Palaobot. Palynol., **143**: 1-81.

Rehder, A. 1903. Synopsis of the genus *Lonicera*. – Ann. Missouri Bot. Gard., **14**: 27-232.

Rehder, A. 1913. *Caprifoliaceae*. – In: **Sargent, C. S.** (ed.), Plantae Wilsonianae: an Enumeration of the Woody Plants Collected in Western China for the Arnold Arboretum of Harvard University during the Years 1907, 1908, and 1910, pp. 106-144, Harvard Univ. Press, Cambridge.

Ronquist, F., Teslenko, M., vander Mark, P., Ayres, D.L, Darling, A., Ho¨hna, S., Larget, B., Liu, L., Suchard, M.A. &Huelsenbeck, J.P. : Mr Bayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. – Syst. Biol., **61**: 539-542.

Sang, T., Crawford, D.J.&Stuessy, T. 1995. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: Implication for biogeography and concerted evolution. – Proc. Natl. Acad. Sci. USA., **92**: 6813-6817.

Shang, X., Pan, H. Li, M. Miao, X. &Ding, H. 2011. *Lonicera japonica* Thunb.: Ethnopharmacology, phytochemistry and pharmacology of an important traditional Chinese medicine. – J. Ethnopharmacol., **138**: 1-21.

Sheidai, M., Zanganeh, S. Haji-ramezanali, R. Nouroozi, M. Noormohammadi, Z. Ghsemzadeh-baraki, S. 2013. Genetic diversity and population structure in four *Cirsium* (*Asteraceae*) species. – Biologia, **68**: 384-397.

Sheidai, M., Ziaee, S., Farahani, F., Talebi, S.Y., Noormohammadi, Z. &Hasheminejad-Ahangarani-Farahani, Y. 2014. Infraspecific genetic and morphological diversity in *Linum album* (*Linaceae*). – Biologia, **69**: 32-39.

Soltis, D.E., Soltis, P.S. 2000. Contributions of plant molecular systematics to studies of molecular evolution. – Plant. Mol. Biol., **42**: 45-75.

Stamatakis, A. 2014. RAxML ver. 8: a tool for phylogenetic analysis and postanalysis of large phylogenies. – Bioinformatics., **30**: 1312-1313.

Swofford, D.L. 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0b10. Sunderland: Sinauer Associates.

Theis, N., Donoghue, J.M. &Li, J. 2008. Phylogenetics of the Caprifolieae and *Lonicera* (Dipsacales) based on nuclear and chloroplast DNA sequences. – Syst. Bot., **33**: 776-783.

Wendelbo, P. 1965. *Caprifoliaceae*. – In: **Rechinger, K.H.,** (ed.), Flora Iranica, 10. Graz: Akademische Druck- u. Verlagsanstalt.

White, T.J., Bruns, T., Lee, S.& Taylor, J. 1990: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. – In: **Innis D.H. Gelfand J.J. Sninsky & al.** (eds). PCR Protocols: a Guide to Methods and Applications. Acad. Press, San Diego: 315-322.

Zeng, H., Li, Y., Chen, J., Wang, X., Qian, Z., Li, Y., Cai, N. & Liu, S. 2017. *Lonicera japonica* 'Fenglei'. – Hort. Sci., **52**: 789-791.