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Revision of *Alnus* (Betulaceae) in Iran using molecular ITS markers and morphological characteristics

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Alnus (Alder) is a genus of Betulaceae, which comprises more than 29 species in the world. According to different classifications of flora, eight taxa of the genus naturally grow in Iran and are distributed along the northern slope of Albourz Mountains in Iran (Hyrcanian forests). In the current research, we investigated morphological characteristics and phylogenetic relationships of 31 populations of the genus in Iran. Twenty-eight qualitative and quantitative traits were studied for morphological evaluation and ITS molecular marker was investigated for molecular study. We used MVSP, SPSS, MrBayes, RAXMLGUI, Mesquite 2.71, Modeltest 3.7 software packages for statistical analyses. Morphological features varied widely among the studied taxa and ANOVA test revealed significant difference for most of them. Moreover, the angle of leaf base, the leaf margin shape, the length and width of the leaf blade, fruits length, and the presence of hair on the petioles, young twigs, and buds were more variable morphological traits. The studied taxa were clustered separately in the UPGMA tree, PCA and PCO plots using morphological traits. In addition, CA-joined plot showed each taxon to have distinct morphological trait(s), which is useful in the identification of the taxa. Phylogeny analysis revealed that *Alnus* genus is a monophyletic group. Furthermore, the studied taxa were clustered separately in phylogenetic dendrogram. According to morphological and ITS data, we listed ten taxa of the genus in Iran and introduced *A. hyrcana* and *A. longiflorescentia* as two new species from Hyrcanian forests of Iran.

Keywords: *Alnus*, Hyrcanian forests, Iran, ITS, new species, morphology

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INTRODUCTION

Alnus Mill. is the genus of Betulaceae and contains about 29–35 species that are mainly distributed across Asia, Africa, Europe, and North America (Murai, 1964; Furlow, 1979; Frodin, Govaers, 1998). Moreover, in Iran, the northern slopes of Albourz Mountains that belong to Euxino-Hyrcanian district are covered with temperate deciduous forests (Zohary, 1973). *Alnus* species forms about 6.7% of these forests in the country (Sagheb-Talebi, 2004).

Sati et al. (2011) believe that several species of the genus are commonly used in traditional medicines. For example, *Alnus hirsuta* Turcz. has been used in oriental medicine as a remedy for fever, haemorrhages, burn injuries, diarrhoea, and alcoholism (Park et al., 2010).

Different studies (Lee et al., 1992; Novaković et al., 2013) proved that several bioactive natural components including diarylheptanoids, polyphenols, flavonoids, terpenoids, and steroids, were isolated from *Alnus* species. Hu and Wang (2011) suggested that diarylheptanoids were considered as the primary bioactive compounds of *Alnus* taxa and attracted attention due to their physiological activities such as anticancer and antioxidative activities.

There are many discussions about the number of *Alnus* species in Iran. For example, Boissier (1879) reported the presence of four taxa including *A. glutinosa* Willd, *A. incana* Willd, *A. cordifoliaa* Ten, and *A. orientalis* Decne in the Flora Orientalis. Browicz (1972) listed three taxa, namely, *A. glutinosa* subsp. *barbata* (C. A. Mey.) Yalt., *A. subcordata* var. *villosa* (Regel) H. J. P. Winkl., and *A. subcordata* C. A. Mey. var. *subcordata* in Flora Iranica.

Moreover, Mobayen (1985) reported the presence of two species, *A. glutinosa* and *A. subcordata*, in Iranian Flora. Kuzeneva (1936) reported 12 taxa of *Alnus* in *Flora U.S.S.R.* More recently, Zare and Amini (2012) introduced five taxa, namely, *A. dolichocarpa* H. Zare, Amini & Assadi, *A. djavanshirii* H. Zare, *A. glutinosa* subsp. *glutinosa* (L.) Gaertn., *A. glutinosa* subsp. *antitaurica* Yalt, and *A. orientalis* Decne from Iran. However, Shayanmehr et al. (2014) doubted the presence of *A. orientalis*, *A. dolichocarpa*, and *A. djavanshirii* in Iran because of morphological similarities to *A. subcordata.*

Hoseinzadeh et al. (2016) investigated the phylogenetic relationships of eight taxa of Iranian *Alnus* using ITS and trnH-psbA. The trnH-psbA chloroplast marker showed lesser separability in comparison with ITS. *A. dolichocarpa*, *A. djavanshirii*, and *A. orientalis* are morphologically very similar to *A. subcordata,* therefore, their taxonomic status is unknown and their presence in the Hyrcanian region is doubted.

It seems that investigation of molecular phylogeny of this genus is a very good and useful approach that may help resolve these taxonomical and evolutionary questions. However, Savard et al., (1993) reported that Betulaceae appeared to be slow-evolving taxa at the DNA level, while the nuclear ribosomal internal transcribed spacer sequences (ITS) were more variable than chloroplast *rbcL* sequences, with potentially useful phylogenetic signal at the genus level. Therefore, in the current study we revised Iranian *Alnus* species using morphological and phylogenetic method (ITS) in order to determine the number of its species in Iran.

MATERIALS AND METHODS

Plant samples

Considering the distribution range of *Alnus* species in Iran, sampling was done in 21 stations from the northern slope of Albourz Mountains at different altitudes of Hyrcanian forests (Table 1). Based on the geographical distribution, one to three populations of each taxon were selected. Plant specimens were identified according to descriptions provided in valuable references such as *Flora Orientalis* (Boissier, 1879), *Flora Iranica* (Browicz, 1972), Flora U.S.S.R (Kuzeneva, 1936), *Flora Turkey* (Yaltrik, 1982) and *Flora of Iran* (Zare, 2016). Herbarium samples were placed in the herbarium of Tehran North Branch, Islamic Azad University (IAUNT).

Table 1. **Locality addresses of the studied *Alnus* samples collected from different localities of Hyrcanian forests**

Taxa	Locality	Geographic coordinates	Elevation (m)	Herbarium no.
A. hyrcana	Golestan-National park	37°23′43.62″N, 55°48′1.5″E	471	16720 IAUNT
A. subcordata var. *subcordata*	Golestan-Ramin	36°59′47.8″N, 55°9′59″E	362	16725 IAUNT
A. subcordata var. *subcordata*	Golestan-Gorgan	36°46′18.17″N, 54°28′3.94″E	477	16727 IAUNT
A. subcordata var. *subcordata*	Mazandaran-Rooyan	36°34′14.38″N, 51°53′3.26″E	–7	16737 IAUNT
A. subcordata var. *villosa*	Mazandaran-Gelvard	36°35′03.80″N, 53°36′23.57″E	753	16732 IAUNT
A. subcordata var. *villosa*	Mazandaran-Sangedeh	36°04′05.63″N, 53°13′01.93″E	1281	16733 IAUNT
A. longiflorescentia	Golestan-Gorgan	36°7′7.7″N, 53°3′20.8″E	640	16719 IAUNT
A. djavanshirii	Mazandaran-Behshahr	36°39′27.87″N, 53°30′26.87″E	443	16729 IAUNT
A. orientalis	Mazandaran-Neka	36°34′15.54″N, 53°29′14.65″E	478	16731 IAUNT
A. orientalis	Mazandaran-Sisangan	36°35′6.3″N, 51° 47′24.36″E	–6	16738 IAUNT
A. glutinosa ssp. *glutinosa*	Mazandaran-Rostam-rood	36°35′35″N, 52° 6′25.18″E	–30	16735 IAUNT
A. glutinosa ssp. *glutinosa*	Mazandaran-Chaloos	36°41′16.82″N, 51°18′29.42″E	–29	16741 IAUNT
A. glutinosa ssp. *glutinosa*	Mazandaran-Chaboksar	36°57′59.5″N, 50°35′12.94″E	–10	16743 IAUNT
A. glutinosa ssp. *antitaurica*	Gilan-Astara	38°25′20″N, 48°52′27.28″E	–22	16750 IAUNT
A. glutinosa ssp. *antitaurica*	Mazandaran-Chaboksar	37°3′54″N, 50°25′25.9″E	–45	16744 IAUNT
A. glutinosa ssp. *antitaurica*	Mazandaran-Mahmood-abad	36°36′45.9″N, 52°12′7.66″E	–20	16734 IAUNT
A. glutinosa ssp. *barbata*	Gilan-Talesh	37°41′3.42″N, 48°59′49.59″E	13	16751 IAUNT
A. glutinosa ssp. *barbata*	Gilan-Astane Ashrafiye	37°14′59.29″N, 49°56’54.65″E	–12	16745 IAUNT
A. glutinosa ssp. *barbata*	Mazandaran-Noshahr	36°38′18.77″N, 51°30′51.23″E	–5	16739 IAUNT
A. dolichocarpa	Gilan-Astara	38°12′33.63″N, 48°53′10.54″E	–10	16749 IAUNT
A. dolichocarpa	Mazandaran-Noshahr	36°38′27.8″N, 51°28′29.96″E	–8	16740 IAUNT

Morphological studies

Measurements of morphological traits were performed on each plant, including its flowering stem. In total, 28 qualitative and quantitative features from both vegetative and reproductive organs were measured on each specimen. Each character was measured four times per each plant sample. Three individuals were studied per each population and the average of each trait was determined. The 17 studied quantitative morphological characteristics were the length and the width of the leaf blade, the length of petiole, the number of lateral vein pairs, the length and width of the male catkin, the number of male catkins on one twig, the length and width of fruits, the length of the pedicel of male and female catkin, the number of fruits (cone) on one twig, the number of teeth in the upper third of the leaf, the distance from the base of the leaf blade to its widest part, the distance from the top of the leaflet to the top of the upper third vein, the angle from the top of the leaf to the top of the upper third nerve, and the angle of the leaf base. In addition, eleven qualitative evaluated features were the leaf base, the apex and margin shape, leaf symmetry, the cone form, the presence of hair on the fruit stalk, annual branch, buds, upper and lower surfaces of leaves, and the branching point.

Statistical analyses

We subjected the quantitative data to one-way analysis of variance (ANOVA) to determine if significant variation existed among the studied taxa for each characteristic measured. Mean and standard deviations of features were calculated. These analyses were performed using SPSS software. Cluster analysis was carried out based on quantitative and qualitative variables using UPGMA tree, PCO, PCA and CA-Joined plots clustering in Multivariate Statistical Package (MVSP) program (Podani, 2000).

DNA extraction, PCR, and sequencing

From the collected plants, three leaf samples were stored in silica gel for molecular study. Genome extraction was performed using the MBST Kit (Shayan et al., 2007) according to the instructions of the manufacturer. PCR was done for DNA amplification in a total volume of 25 µl (3 µl DNA sample, 12.5 µl Master Mix RED, and 0.5 µl of forward and reverse primers, 1 µl DMSO and 7.5 µl deionized water (Table 2). PCR product was tested by 0.1 agarose gel. Sequencing and purification of PCR product were carried out by Sectech Co.

Phylogenetic analysis

Sequences processing was performed with Sequencer 4.1.4 software. *Betula pendula* Roth was considered as out group (its sequence was selected in the GenBank) (Table 3). The sequences were aligned using the Mesquite 2.71 software. The Nexus file was analyzed with Modeltest 3.7 software. The best evolutionary model for the tenth generation was selected and analyzed. The final cladogram was drawn with MrBayes 3.1.2 and raxmlGUI software.

Table 2. **Sequences of ITS primers**

Primer name	Primer sequences	PCR conditions	References
AB101	5′-ACGAATTCATGGTCCGGT-GAAGTGTTCG-3′	95°C 5′; 35cycles; 95°C 30″; 57.5°C 30″; 72°C 1.5′; 72°C 7′	Douzery et al., 1999
AB102	5′-TAGAATTCCCCGGTTCGCTC-GCCGTTAC-3′		

Table 3. **GenBank accession numbers used in the molecular analysis of the ITS marker**

Species	GenBank accession numbers	Species	GenBank accession numbers
A. mandschurica	AY352315	*A. nepalensis*	AY352318
A. sinuata	AY352325	*A. ferdinandi-coburgii*	FJ825416
A. maximowiczii	AB243877	*A. cremastogyne*	FJ825413
A. fruticosa	AY352309	*A. cordata*	AJ251663
A. crispa	AJ251681	*A. trabeculosa*	FJ825408
A. viridis	AY352329	*A. henryi*	GU112748
A. pendula	AJ251682	*A. oblongifolia*	AY352319
A. sieboldiana	FJ825384	*A. rhombifolia*	AJ251669
A. firma	FJ825381	*A. acuminata*	AF432066
A. formosana	AJ251678	*A. jorullensis*	AJ251672
A. maritima	AJ251679	*A. matsumurae*	FJ825397
A. serrulatoides	FJ825428	*A. tenuifolia*	AJ251666
A. fauriei	FJ825433	*A. rugosa*	AY352313
A. japonica	FJ825425	*A. hirsuta*	FJ825389
A. serrulata	AY352322	*A. incana*	AJ251665
A. nitida	AJ783638	*A. rubra*	FJ825402
Betula pendula	AY352332		

RESULTS

Morphological analysis

The mean and standard deviation of some quantitative morphological variables are presented in Table 4. Morphological characteristics highly varied among the studied taxa. For example, the smallest and the largest blade leaf length was recorded in *A. glutinosa* subsp. *antitaurica* and *A. djavanshirii*, respectively. The narrowest blade leaf was found in *A. subcordata* var. *villosa*, while the broadest one was observed

Table 4. **Mean and standard deviation of some important quantitative morphological features (all values are in cm)**

Taxa		Blade leaf length	Blade leaf width	Petiole length	Number of lateral vein pairs	Number of female catkins	Female catkin length	Female catkin width	Female catkin pedicle length	Number of male catkins	Male catkin length	Male catkin width	Male catkin pedicle length
A. subcordata var. *villosa*	Mean	8.90	5.50	2.80	11.00	2.00	2.10	1.3	0.9	4.33	7.24	0.29	1.84
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. dev.	0.10	0.50	0.50	1.00	.00	.20	1.00	1.00	0.53	.74	1.12	0.64
A. glutinosa ssp. *glutinosa*	Mean	9.70	7.03	3.33	10.00	3.00	2.93	0.9	1.5	4.33	7.50	0.3	1.93
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. dev.	1.20	.15275	.11	.00	1.00	.05	.00	2.00	0.57	.700	1.00	0.57
A. glutinosa ssp. *antitaurica*	Mean	8.83	6.43	1.80	10.00	6.66	1.33	0.73	1.9	4.00	3.50	0.5	1.4
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. dev.	0.35	.15	.36	.00	1.52	0.05	0.57	4.00	0.00	.00	2.00	1.00
A. longiflorescentia	Mean	14.40	8.70	3.30	11.00	3.00	3.40	1.1	2.0	4.00	4.30	0.3	1.0
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. dev.	1.80	.80	.20	1.00	1.00	.80	1.00	8.00	0.00	.40	1.00	.00
A. hyrcana	Mean	11.05	7.05	2.150	11.50	2.00	2.25	1.4	1.2	4.00	2.40	.35	1.1
	N	2	2	2	2	2	2	2	2	2	2	2	2
	Std. dev.	0.21	0.21	0.35	.70711	0.00	0.07	0.00	1.41	0.00	0.14	0.70	0.00
A. orientalis	Mean	12.70	6.40	2.80	11.00	2.00	2.00	1.1	0.9	4.00	2.80	.3	0.9
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. dev.	0.50	1.20	.20	.00	1.00	.100	1.00	1.00	0.00	0.80	1.00	2.00
A. subcordata var. *subcordata*	Mean	11.50	5.70	2.70	11.00	3.00	2.90	1.6	1.7	4.00	6.00	.4	1.5
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. dev.	1.50	0.70	0.50	3.0	1.00	0.40	1.00	2.00	1.00	0.40	0.00	0.00
A. djavanshirii	Mean	14.50	7.70	3.10	10.00	3.00	2.20	0.9	1.3	3.99	5.84	.38	1.3
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. dev.	0.500	.100	0.10	0.00	0.00	0.0	0.00	2.00	1.00	0.42	0.07	0.01
A. dolichocarpa	Mean	13.10	9.30	2.70	11.00	3.00	2.80	1.5	1.8	3.00	6.00	.4	0.9
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. dev.	2.60	0.80	0.20	1.00	0.00	0.80	0.00	2.00	1.00	0.00	0.00	1.00
A. glutinosa ssp. *barbata*	Mean	10.60	7.10	3.03	10.00	5.66	1.80	1.0		6.33	4.80	.3	1.8
	N	3	3	3	3	3	3	3		3	3	3	3
	Std. dev.	0.10	0.10	0.05	0.00	1.52	0.20	1.15		0.57	0.10	0.00	2.00

in *A. dolichocarpa*. The shortest and the largest petioles were registered in *A. glutinosa* subsp. *antitaurica* and *A. longiflorescentia*. The biggest number of female catkins (6.66) belonged to *A. glutinosa* subsp. *antitaurica,* but most of the studied taxa had two and three female catkins. *A. longiflorescentia* had the longest female catkin, while *A. glutinosa* subsp. *antitaurica* had the shortest female catkin. Moreover, the widest female catkin was recorded in *A. subcordata* var. *subcordata*, but *A. glutinosa* subsp. *antitaurica* had the narrowest female catkin.

The ANOVA test revealed significant difference for all quantitative morphological characteristics, except for the width of the male catkin and the number of lateral vein pairs (Table 5).

The studied taxa separated from each other and were clustered separately in the UPGMA tree of morphological data (Fig. 1); moreover, PCO and PCA plots (Fig. 2, 3) produced similar outputs. Therefore, taxa arrangements in the tree were discussed here: the tree had two branches. The small branch divided into two sub-branches. *A. djavanshirii* were placed in a sub-branch, while three varieties of *A. subcordata* existed in another sub-branch, which had two groups. *A. glutinosa* subsp. *glutinosa* and

Table 5. **Results of the ANOVA test among the studied quantitative features**

		Sum of squares	Difference	Mean square	F	Sig.
Blade leaf length	Between groups	118.340	9	13.149	8.701	0.0
	Within groups	28.712	19	1.511		
	Total	147.052	28			
Blade leaf width	Between groups	39.660	9	4.407	11.795	0.0
	Within groups	7.098	19	.374		
	Total	46.759	28			
Petiole length	Between groups	5.940	9	.660	7.472	0.0
	Within groups	1.678	19	.088		
	Total	7.619	28			
Number of lateral vein pairs	Between groups	8.328	9	.925	.718	0.687
	Within groups	24.500	19	1.289		
	Total	32.828	28			
Number of female catkins	Between groups	65.494	9	7.277	7.977	0.0
	Within groups	17.333	19	.912		
	Total	82.828	28			
Female catkin length	Between groups	10.475	9	1.164	7.184	0.0
	Within groups	3.078	19	.162		
	Total	13.553	28			
Female catkin width	Between groups	211.908	9	23.545	39.473	0.0
	Within groups	11.333	19	.596		
	Total	223.241	28			
Female catkin pedicle length	Between groups	406.615	8	50.827	4.364	0.005
	Within groups	198.0	17	11.647		
	Total	604.615	25			
Male catkin width	Between groups	11.152	7	1.593	1.648	0.197
	Within groups	14.500	15	.967		
	Total	25.652	22			
Male catkin length	Between groups	59.815	7	8.545	43.597	0.0
	Within groups	2.940	15	.196		
	Total	62.755	22			
Male catkin pedicle length	Between groups	367.942	7	52.563	38.151	0.0
	Within groups	20.667	15	1.378		
	Total	388.609	22			
Number of male catkin	Between groups	18.580	7	2.654	7.465	0.001
	Within groups	5.333	15	.356		
	Total	23.913	22			

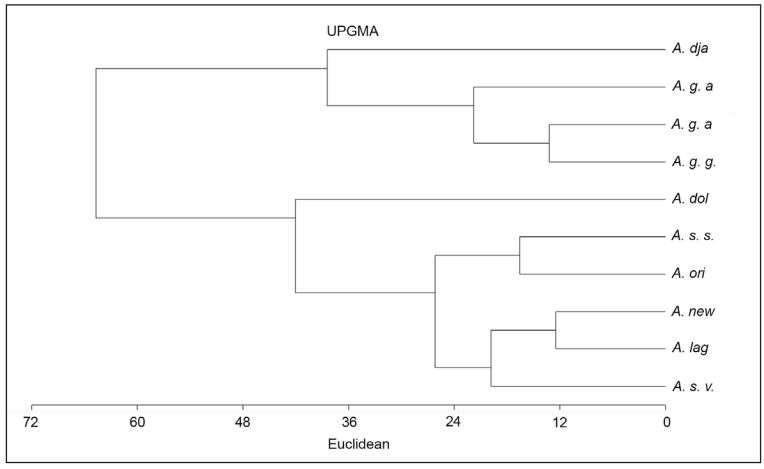

Fig. 1. The UPGMA tree of the studied taxa based on morphological traits. Abbreviations: *A. g.g* = *A. glutinosa* subsp. *glutinosa*; *A. g.b* = *A. lutinosa* subsp. *barbata*; *A. g.a* = *A. glutinosa* subsp. *antitaurica*; *A. s.s* = *A. subcordata* var. *subcordata*; *A. s.v* = *A. subcordata* var. *villosa*; *A. ori* = *A. orientalis*; *A. dol* = *A. dolichocarpa*; *A. dja* = *A. djavanshirii*; *A. lag* = *A. longiflorescentia*; *A. New* = *A. hyrcana*

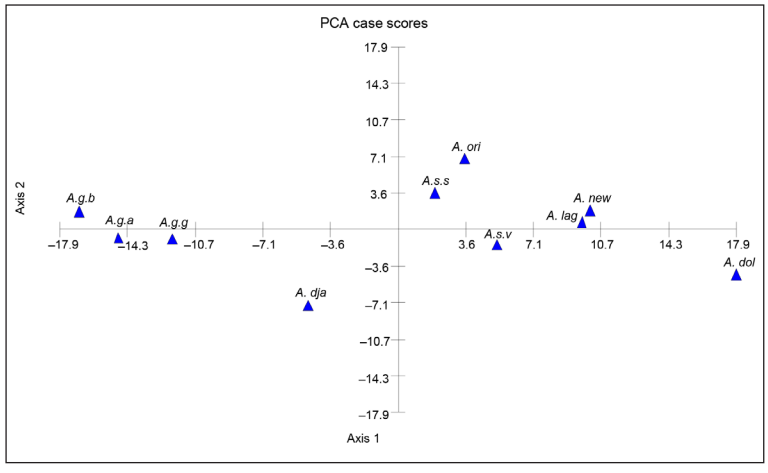

Fig. 2. PCA plot of the studied taxa based on morphological variables. Abbreviations: *A. g.g* = *A. glutinosa* subsp. *glutinosa*; *A. g.b* = *A. glutinosa* subsp. *barbata*; *A. g.a* = *A. glutinosa* subsp. *antitaurica*; *A. s.s.* = *A. subcordata* var. *subcordata*; *A. s.v* = *A. subcordata* var. *villosa*; *A. ori* = *A. orientalis*; *A. dol* = *A. dolichocarpa*; *A. dja* = *A. djavanshirii*; *A. lag* = *A. longiflorescentia*; *A. New* = *A. hyrcana*

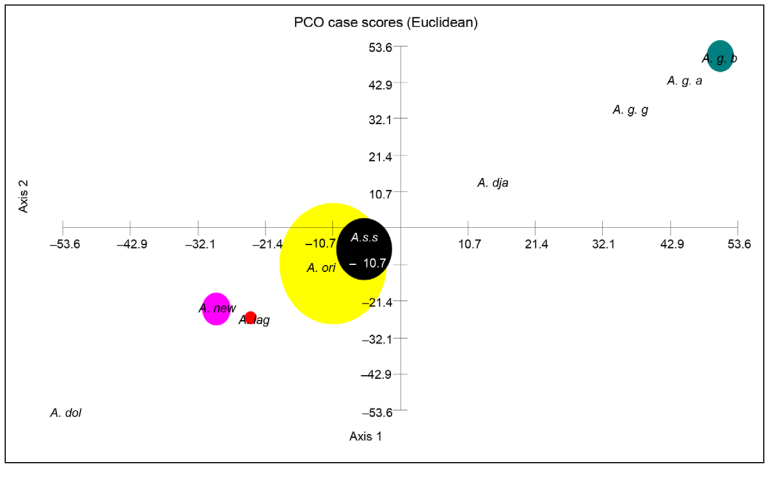

Fig. 3. PCO plot of the studied taxa based on morphological characteristics. Abbreviations: *A. g.g* = *A. glutinosa* subsp. *glutinosa*; *A. g.b* = *A. glutinosa* subsp. *barbata*; *A. g.a* = *A. glutinosa* subsp. *antitaurica*; *A. s.s* = *A. subcordata* var. *subcordata*; *A. s.v* = *A. subcordata* var. *villosa*; *A. ori* = *A. orientalis*; *A. dol* = *A. dolichocarpa*; *A. dja* = *A. djavanshirii*; *A. lag* = *A. longiflorescentia*; *A. New* = *A. hyrcana*

A. glutinosa subsp. *antitaurica* formed a group and *A. glutinosa* subsp. *barbata* joined them.

In another branch, we found two sub-branches: one of them was small and contained *A. dolichocarpa*, while another sub-branch was large and consisted of two groups. *A. subcordata* var. *subcordata* and *A. orientalis* clustered as a group, but the other group had *A. longiflorescentia* and *A. hyrcana* as a group and *A. subcordata* var. *villosa* joined them.

CA-joined plot proved that each taxon had distinct morphological feature(s), which was useful in identification of them. For example, presence of indumentum on petiole, bud and

leaf were prominent traits for *A. hyrcana* and angle of leaf base and female catkin width for *A. longiflorescentia* (Fig. 4).

Molecular analysis

The results of molecular analysis given by the Bayesian method, confirmed that *Alnus* genus is a monophyletic group and that there were two separate clades (I and II) for this genus in Iran (Fig. 5). The main clade I is divided into sub-clades A and B. Sub-clade A includes *A. glutinosa* subspecies (including *A. glutinosa* subsp. *glutinosa*, *A. glutinosa* subsp. *barbata*, and *A. glutinosa* subsp. *antitaurica*)

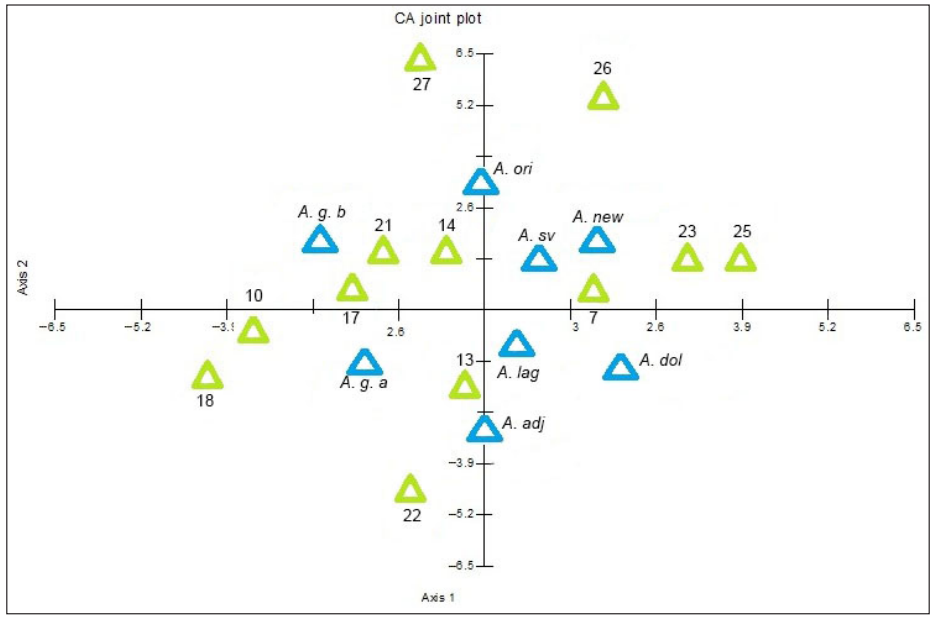

Fig. 4. CA-joined plot of the studied taxa and their morphological variables. Abbreviation: *A. g.g* = *A. glutinosa* subsp. *glutinosa*; *A. g.b* = *A. glutinosa* subsp. *barbata*; *A. g.a* = *A. glutinosa* subsp. *antitaurica*; *A. s.s* = *A. subcordata* var. *subcordata*; *A. s.v* = *A. subcordata* var. *villosa*; *A. ori* = *A. orientalis*; *A. dol* = *A. dolichocarpa*; *A. dja* = *A. djavanshirii*; *A. lag* = *A. longiflorescentia*; *A. New* = *A. hyrcana*

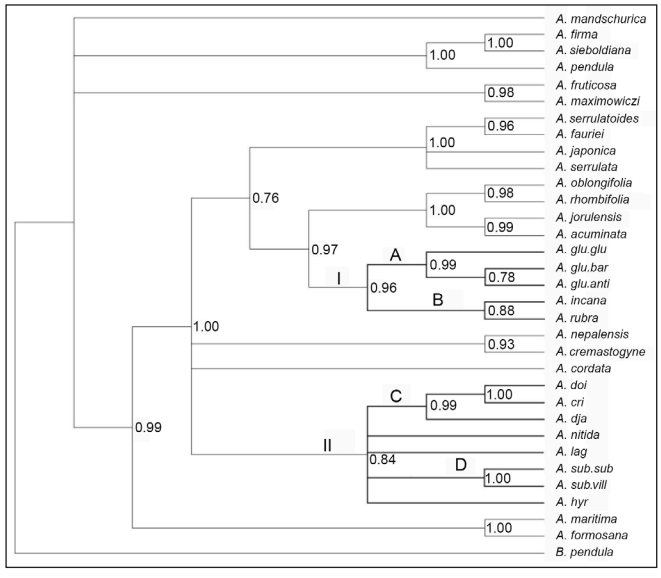

Fig. 5. Cladogram resulting from molecular analysis (Bayesian method). Abbreviations: *A. glu.glu* = *A. glutinosa* subsp. *glutinosa*; *A. glu.bar* = *A. glutinosa* subsp. *barbata*; *A. glu.anti* = *A. glutinosa* subsp. *antitaurica*; *A. sub.sub* = *A. subcordata* var. *subcordata*; *A. sub.vill* = *A. subcordata* var. *villosa*; *A. ori* = *A. orientalis*; *A. doli* = *A. dolichocarpa*; *A. dja* = *A. djavanshirii*; *A. lag* = *A. longiflorescentia*; *A. hyr* = *A. hyrcana*

and sub-clade B includes *A. incana* and *A. rubra* (not native to Iran) with a statistical support of 0.96. The main clade II is also divided into sub-clades D and C. Sub-clade C contains *A. orientalis*, *A. dolichocarpa*, and *A. djavanshirii* (with a statistical support of 0.88). *A. djavanshirii* was also found to be close to *A. orientalis* and *A. dolichocarpa* (with a statistical support of 0.99). Sub-clade D contained *A. subcordata* var. *subcordata* and *A. subcordata* var. *villosa* (with a statistical support of 0.89). Two new species, *A. hyrcana* and *A. longiflorescentia*, were clustered far from the other taxa. In addition, molecular analysis using the raxml method also indicated that *Alnus* was a monophyletic group and *Alnus* taxa of Iran were placed into two separate clades (I and II) (Fig. 6). Clade I includes *A. subcordata* var. *villosa*, *A. subcordata* var. *subcordata*, *A. orientalis*, *A. dolichocarpa*, and *A. djavanshirii*. Two new species, *A. hyrcana* and *A. longiflorescentia*, were placed far from the other taxa. *A. glutinosa* subsp. *glutinosa*, *A. glutinosa* subsp. *barbata*, and *A. glutinosa* subsp. *antitaurica* were grouped in clade II.

Descriptions of new species

Based on the results of morphological and phylogenetic studies, two new species were identified in Iran. The main morphological differences between these new species with other closet species of *Alnus* are listed in Table 6. The morphology and geographical details of these species were given here:

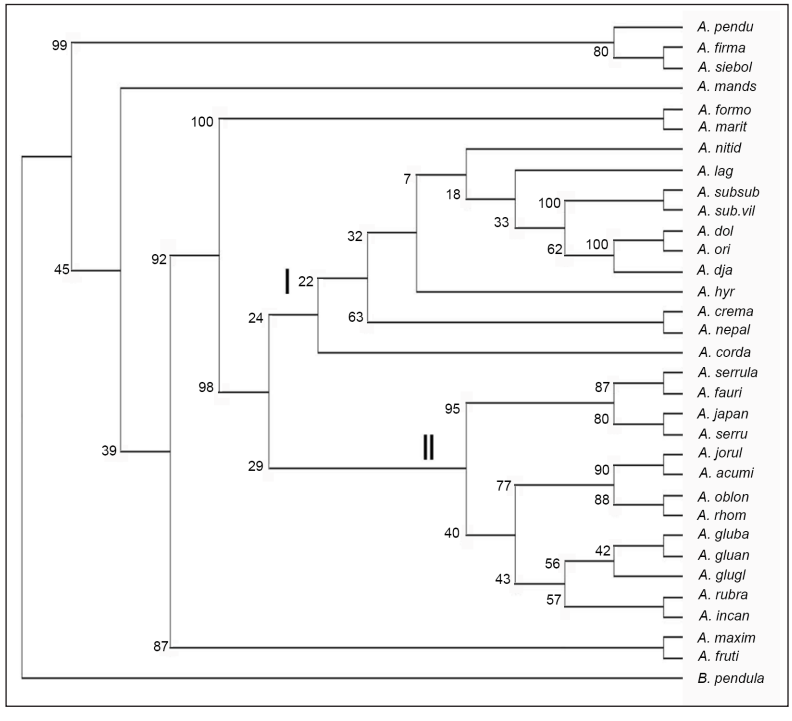

Fig. 6. Cladogram resulting from molecular analysis (RAXML method). Abbreviations: *A. glu.glu* = *A. glutinosa* subsp. *glutinosa*; *A. glu. bar* = *A. glutinosa* subsp. *barbata*; *A. glu.anti* = *A. glutinosa* subsp. *antitaurica*; *A. sub.sub* = *A. subcordata* var. *subcordata*; *A. sub. vill* = *A. subcordata* var. *villosa*; *A. ori* = *A. orientalis*; *A. doli* = *A. dolichocarpa*; *A. dja* = *A. djavanshirii*; *A. lag* = *A. longiflorescentia*; *A. hyr* = *A. hyrcana*

Table 6. **Comparison between the new species of *Alnus* and the species close to them**

Characters	*A. hyrcana*	*A. longiflorescentia*	*A. dolichocarpa*	*A. orientalis*	*A. subcordata* var. *villosa*
Braches indumentum	Pubescent	Glabrous	Puberulent	Glabrous	Puberulent
Leaf base angle	125	124	150	104	112
Leaf margin shape	Serrulate	Large lobed dentate	Double serrate	Large lobed dentate	Serrate
Leaf blade length	10.9 cm	14.4 cm	13.1 cm	12.7 cm	8.9 cm
Leaf blade width	6.9 cm	8.7 cm	9.3 cm	6.4 cm	5.5 cm
Cone length	2.3 cm	3.4 cm	2.8 cm	2 cm	2.1 cm

Alnus hyrcana: Sharifnia & Gholamiterojeni, sp. nova (Figs. 7 and 8).

Type: Iran, Golestan National Park, 37°23′43.62″N, 55°48′1.5″E, 471m, 20 October 2015 Sharifnia & Gholamiterojeni 16720 (holotype IAUNT).

A tree of about 10 m in height, deciduous, young twigs and buds tomentose. Leaves ovate, 11.2–16.2 cm long and 10.6–11.2 cm broad, acute, semi-cordate at the base, serrulate at the margin, with 10–12 pairs of veins, tomentose on the upper surface, more densely hairy on the lower surface; petioles 1.5–2.4 cm long, pubescent. Male inflorescence cluster with 4 catkins, 1.8–2.8 cm long and 3–4 mm wide; stalk 0.5–1.2 cm long. Female inflorescence cluster with 2–3 cone-like catkins; catkins ovate, 2.2–2.4 cm long and 1.4–1.5 cm wide; peduncle 1–1.3 cm long. Seed winged.

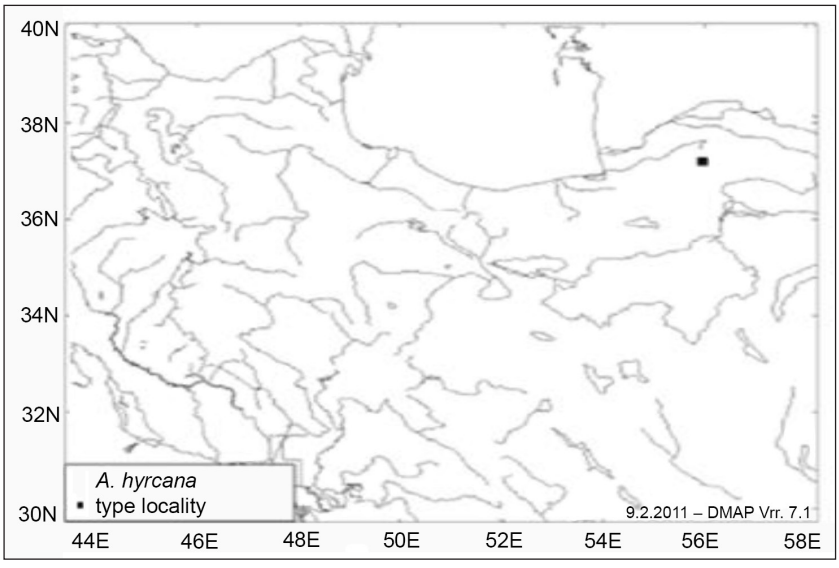

Fig. 7. Distribution map of *Alnus hyrcana* in Iran

Fig. 8. *A. hyrcana*. a: male catkins. b: female cone-like catkin. c: bud

Alnus longiflorescentia: Sharifnia & Gholamiterojeni, sp. nova (Figs. 9 and 10).

Type: Iran, Golestan Province, Gorgan, Ziarat, 36°7′7.7″N, 53°3′20.8″E, 640 m, 16 September 2015 Sharifnia & Gholamiterojeni 16719 (holotype IAUNT).

A tree of about 10 m in height, deciduous, young twigs; buds and petioles puberulent. Leaves elliptic-ovate, 11.2–16.2 cm long and 6.8–9.8 cm wide, usually long-acuminate at apex, asymmetric cordate to cuneate at the base, at the margin with large teeth in the upper third

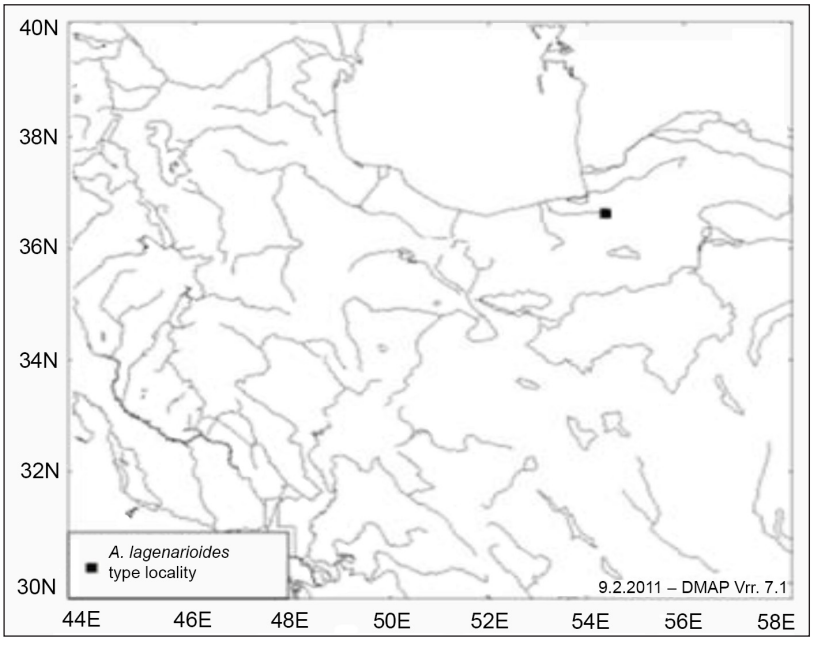

Fig. 9. Distribution map of *A. longiflorescentia* ın Iran

Fig. 10. *Alnus longiflorescentia*. a: male catkins. b: female cone-like catkin. c: bud

of the upper half, with sparse hairs on the upper surface, glabrous on the lower surface with less hairs on the veins and presence of tufts of hairs in the axil of veins, with 9–14 pairs of lateral veins; petioles with long hairs, 1.5–2.4 cm long. Male inflorescence clusters with 4–5 catkins, catkins 3–4.7 cm long and 3–4 mm wide; stalk 0.5–1 cm long. Female inflorescence large, cone-like, cylindrical, 2.5–4.2 cm long and 1–1.3 cm wide; peduncle 1–2.8 cm long. Seeds winged.

DISCUSSION

In the current study, we used morphological and molecular data for the delimitation of species in Iranian *Alnus* species. For morphological study, 28 distinct characteristics were studied; the selection of these features was based on previous morphological studies of the genus (Chang et al., 2005; Banaev, Bazunt, 2007; Eom et al., 2011; Basic et al., 2014; Poljak et al., 2014).

The ANOVA test revealed significant differences for most morphological characteristics. The obtained results of clustering morphological study showed that ten taxa of seven *Alnus* species grow naturally in Iran. Some of them (*A. glutinosa* and *A. subcordata*) have infraspecific taxonomic ranks: *A. glutinosa* subsp. *antitaurica*, *A. glutinosa* subsp. *barbata*, *A. glutinosa* subsp. *glutinosa*, *A. subcordata* var. *villosa*, and *A. subcordata* var. *subcordata*, while the rest – *A. dolichocarpa*, *A. djavanshirii*, and *A. orientalis* – are monotypic. In addition, two species, *A. hyrcana* and *A. longiflorescentia*, are introduced as new species from Iran.

The new species were compared morphologically with other Iranian taxa of the genus. This comparison showed differences in some characteristics including the angle of the leaf base, leaf margin, the length of leaf blade, the width of leaf blade, the length of the female catkin, and the presence of hair on young twigs.

We compared these new species (*A. hyrcana* and *A. longiflorescentia*) with other similar Iranian taxa and found that *A. longiflorescentia* differed from other species by bigger female inflorescence. Beside, *A. hyrcana* is separated from the other species by the largest amount of hair on young twigs, buds, leaves, and petioles. *A. orientalis* and *A. djavanshiri* are morphologically close to *A. subcordata*.

For phylogeny study, we selected the ITS marker, because previous studies revealed that ITS was a good phylogeny marker for the genus. For example, Chen and Li (2004) used ITS sequences to assess the phylogenetic relationships of *Alnus* species. Moreover, Ren et al. (2010) used the nuclear ITS marker and three chloroplastic markers (rbc L, mat K, trnH- psbA) to assay investigate 23 *Alnus* species throughout the world. Results of their study showed that the separability of rbcL, matK, trnH-psbA, and ITS in the species level was 10%, 31.25%, 63.6% and 76.9%, respectively. Therefore, the ITS sequence is the most powerful molecular marker to identify *Alnus* species.

There are many discussions about the taxonomic status of genus *Alnus*. Linnaeus (1753) considered *Alnus* as part of genus *Betula*, but Murai (1964) identified *Alnus* as a genus that is distinct from *Betula*. Several morphological (Crane, 1989; Furlow, 1990) and molecular (Bousquet et al., 1992; Savard et al., 1993) studies confirmed separation of *Alnus* from *Betula*.

Based on the phylogeny analyses, we found that *Alnus* taxa were a monophyletic group and genus *Betula* could be considered as a sister group for *Alnus*. Our findings agreed with a previous study of Navarro et al. (2003). They evaluated phylogeny of 19 species of *Alnus* and found that all species together made a monophyletic group close to *Betula*.

The studied taxa were separated in Bayesian cladogram of ITS data and created similar results with morphological investigation. Furthermore, ITS data confirmed the presence of two new species (*A. hyrcana* and *A. longiflorescentia*) in Iran, therefore it is clear that 10 taxa of Alder species occur in the country's Hyrcanian forest. These results agreed with Zare and Amini (2012) study and proved the existence of *A. dolichocarpa*, *A. orientalis*, and *A. djavanshirii* in Iran, and contradicted some similar previous studies, such as Browics (1972) and Hoseinzadeh et al. (2016).

CONCLUSIONS

Alnus is a problematic genus of Betulaceae, which grow widely in different parts of the world, including Iran. There are many discussions about the number of species of the genus in the country and therefore we conducted morphological and molecular ITS studies for the delimitation of species of *Alnus* in Iran. A morphological study proved the existence of ten taxa in Iran, with two new species. Moreover, molecular phylogenetic data revealed the genus is monophyletic and had ten taxa, including five monotypic species and five species with infraspecific ranks, three subspecies and two varieties. The new species had prominent morphological traits, which were useful in species identification.

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BERŽINIŲ GENTIES (BETULACEAE) JUODALKSNIO (*ALNUS*) APŽVALGA IRANE NAUDOJANTIS MOLEKULINIAIS ITS ŽYMENIMIS IR MORFOLOGINĖMIS CHARAKTERISTIKOMIS

Santrauka

Alksniai (*Alnus*) yra beržinių (Betulaceae) genties augalai, pasaulyje jų yra daugiau nei 29 rūšys. Irane aptinkami aštuoni šiaurinėje Elburso kalnyno dalyje, Hirkanijos miškuose, natūraliai augantys šios genties taksonai. Tyrimo metu analizuotos morfologinės charakteristikos ir filogenetiniai ryšiai tarp 31 Irane augančios alksninių genties populiacijos. Morfologinei analizei buvo pasitelkti ITS regiono žymenys – tirtos 28 kokybinės ir kiekybinės žymės. Statistinei analizei naudotos MVSP, SPSS, MrBayes, RAXMLGUI, Mesquite 2.71 ir Modeltest 3.7 kompiuterinės programos. Tirtų taksonų morfologinės savybės itin varijavo – ANOVA testas atskleidė statistiškai reikšmingus skirtumus. Labiausiai skyrėsi lapo pagrindo kampas, lapo forma ir ilgis, lapo galo plotis, vaisių ilgis, lapkočio, jaunų šakelių bei pumpurų plaukuotumas. Analizuoti taksonai buvo išskirstyti pagal PCA ir PCO morfologinius žymenis. CA jungimas rodo, kad kiekvienas taksonas turi specifinę morfologinę savybę, naudingą identifikavimui. Filogenetinė analizė atskleidė, kad alksninių gentis yra monofilinė; sudaryta taksonų filogenetinė dendrograma. Remiantis gautais morfologiniais duomenimis bei ITS rezultatais, Irane paplitusi alksninių gentis suskirstyta į 10 taksonų ir įtrauktos dvi naujos rūšys – *A. hyrcana* ir *A. longiflorescentia*, aptiktos Hirkanijos miškuose.

Raktažodžiai: *Alnus*, Hirkanijos miškai, Iranas, ITS, naujos rūšys, morfologija