J Genet Resour2022;8(1): 69-80 *Homepage: http://sc.journals.umz.ac.ir/*

RESEARCH ARTICLE *DOI: 10.22080/jgr.2021.22257.1277*

J|G|R

Systematics of *Carpinus*: Molecular Phylogeny and Morphology

Afsaneh Riahee[1], Mostafa Assadi[2]*, Habib Zare[3] and Iraj Mehregan[1]

[1] Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2] Research Institute of Forests and Rangelands, Agricultural Research Education and Extension Organization, Tehran, Iran
[3] Herbarium of Nowshahr, Botanical Garden, Agricultural and Natural Resources Center of Mazandaran, Nowshahr, Iran

ARTICLE INFO

Article history:
Received 25 July 2021
Accepted 25 November 2021
Available online 12 December 2021

Keywords:
Carpinus
Hyrcanian forests
Iran morphology
ITS region

Corresponding author:
✉ M. Assadi
assadi@rifr-ac.ir

p-ISSN 2423-4257
e-ISSN 2588-2589

ABSTRACT

Carpinus is a genus of the family Betulaceae that comprises 42 species worldwide. Moreover, more than a third of total Caspian forests are occupied by *C. betulus* and it has an important role in slope stabilizing. *C. orientalis*, commonly known as oriental hornbeam, is a small tree or often shrub, rarely over 10 m tall, and a major pioneer species on slopes in shallow humus-poor or rocky soils. This species is distributed from southeastern Europe to the north of Iran, from west to easternmost of the Hyrcanian forest. Due to their peculiar and beautiful fruit cluster, some hornbeams are used as important ornamental plants. Taxonomy of this genus has always been problematic in Iran and the number of species ranges from 2 to 4 in different taxonomic literature. In the current study, we applied morphometric (PCA and cluster analyses) and molecular (ITS region) approaches to delineate the species boundary of the genus in Iran. Thirty-six quantitative and qualitative characters were used for morphological analyses. The PCA plot of morphological data divided the studied population into three groups. However, the cluster analysis revealed two major groups. Moreover, Iranian species of the genus *Carpinus* formed two distinct clades in the molecular analyses. The results of the present study showed that there are two *Carpinus* species in Iran, including *C. betulus* and *C. orientalis* with two subspecies and *C. schuschaensis* is introduced as a synonym for *C. orientalis* subsp. macrocarpa. In addition, the intraspecific morphological diversity has blurred species boundaries.

Please cite this paper as: Riahee A, Assadi M, Zare H, Mehregan I. 2022. Systematics of *Carpinus*: molecular phylogeny and morphology. *J Genet Resour* 8(1): 69-80. doi: 10.22080/jgr.2021.22257.1277.

Introduction

Carpinus L. (Linnaeus, 1960), with about 42 species, is a subfamily of Coryloideae Betulaceae (Yoo and Wen, 2007; WCSP, 2016; Holstein and Weigend, 2017; Li *et al.*, 2018;) The genus has an intercontinental disjunctive distribution in eastern Asia (ca. 30 species), North America (2 species) and Europe (2 species, Jeon *et al*., 2007). China, with 28 endemic species, is the place of central diversity of the genus *Carpinus* (Li and Skvortsov, 1999). Due to their peculiar and beautiful fruit clusters, some hornbeams are used as important ornamental plants (Fini and Ferrini, 2011; Li *et al*., 2018). *C. betulus* L. and *C. orinetalis* Mill. are the two main species of *Carpinus* in Iran (Browicz, 1972). The first species is a medium-sized deciduous tree with a height of 20-30 m, and semi-shade tolerance in the Hyrcanian forest. Its altitudinal distribution ranges from the sea level to 2300 m in Golestan, Gilan, Mazandaran, Northern Khorassan, Azerbaijan (Browicz, 1972), and Semnan Provinces (Mozaffarian, 2005). Moreover, more than a third of total Caspian forests are occupied by *C. betulus* and it has an important role in slope stabilizing (Abdi *et al*., 2009). *C. orientalis*, commonly known as oriental hornbeam, is a small tree or often shrub, rarely over 10 m tall, and a major pioneer species on slopes in shallow humus-poor or rocky soils (Bergmeier and Dimopoulos, 2008; Čarni *et al*., 2009). This

species is distributed from southeastern Europe to the north of Iran, from west to easternmost of the Hyrcanian forest (Browicz, 1972; Colagar *et al*., 2015; Razaz *et al*., 2015). The taxonomy of this genus in Iran is mainly based on morphological characters. However, intraspecific morphological variation and the presence of intermediate phenotypes due to diversity of habitat and possible hybridization have led to different taxonomic classifications of the genus. Consequently, there is a disagreement among researchers about the number of species in Iran (Browicz, 1972; Mobayen, 1979; Sabety, 2001). Browicz in Flora Iranica (1972) classified the genus of *Carpinus* in two species, *C. betulus* with two varieties (*C. betulus*. var. *betulus* and *C. betulus* var. *parva* Radde-Fomin), and *C. orientalis* with two subspecies (*C. orientalis* subsp. *macrocarpa* (Willk.) Browicz and *C. orientalis* subsp. *orientalis*), as well as a hybrid taxon, *C. betulus* × *orientalis*; ×*C. schuschaensis* Winkl (Table 1). Mobayen (1979) reported the presence of three species, including *C. betulus*, *C. orinetalis*. and *C. schuschaensis* H. Winkl. from the Hyrcanian forest of Iran (Table1). Sabety (2001) described four taxa, namely *C. betulus*, *C. orinetalis*, *C. schuschaensis* H. Winkl., *C. macrocarpa* Wilk. and divided the first species into four varieties, *C. betulus* var. *betulus* Browicz, *C. betulus* var. *carpinizza* (Host) Neilr, *C. betulus* var. *parva* Radde-Fomin. Sabety (2001) treated *C. orientalis* subsp. *macrocarpa* as a distinct species, *C. macrocarpa* (Table 1). The taxonomic treatment of Mozaffarian (2005) was similar to Browicz (1972). However, he did not mention *C. schuschaensis* for the flora of Iran (Table 1).

Table 1. Summary of the taxonomical history of *Carpinus*.

Researchers	Number of species in Iran	Taxa
Browicz	3 species	*C. betulus*. var. *betulus* *C. betulus* var. *parva* Radde-Fomin *C. orientalis* subsp. *macrocarpa* (Willk) *C. orientalis* subsp. *orientalis* *C. betulus*×*orientalis*; × *C. schuschaensis*
Mobayen	3 species	*C. betulus*, *C. orinetalis*. *C. schuschaensis*
Sabety	4 taxa	*C. betulus* var. *betulus* Browicz *C. betulus* var. *carpinizza* (Host) Neilr, *C. betulus* var. *parva* Radde-Fomin. Sabety *C. orinetalis*, *C. schuschaensis* *C. macrocarpa*
Mozaffarian	2 species	*C. betulus* var. *betulus* Browicz *C. betulus* var. *parva* Radde-Fomin. Sabety *C. orientalis* subsp. *macrocarpa* (Willk) *C. orientalis* subsp. *Orientalis*

In the last few decades, DNA markers have been used as an effective technique for resolving taxonomic status of species difficult to identify by morphological characters (Kress and Erickson, 2007). The nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS) is one of the most important DNA barcodes for species identification, even in closely related taxa (Alvarez and Wendel, 2003; Small *et al*., 2004; Soltis *et al*., 2004; Kress *et al*., 2005; Yousefzadeh *et al*., 2012; Zhang *et al*., 2012; Yousefzadeh *et al*., 2019). In this regard, the present study aims to delineate the number of Iranian *Carpinus* species using morphological and molecular (ITS region) methods.

Materials and Methods

Plant materials

In the current study, 85 individuals belonging to 17 populations of the genus *Carpinus* were sampled between March and August of 2018 from the Hyrcanian forest of Iran (Golestan, Gilan, Mazandaran, and Western Azerbaijan Provinces) and the lowland of Caspian plains to highland forests at the northern slope of Alborz Mountains (Table 2, Fig. 1).

Table 2. Location and herbarium accession numbers of the studied populations of *Carpinus* species collected by Riyahee in Iran.

Species	Pop	Locality	Latitude	Longitude	Altitude (m)	Herbarium ID
C. betulus var. *parva*	1	Mazandaran, Chamestan toward Waz forest	36°01' 11"	52°13'26"	174	IAUH-000014976
C. betulus var. *betulus*	2	Mazandaran, Kheyroud-Kenar forest	36˚54' 12"	51˚59'27"	118	IAUH-000014977
C. betulus var. *betulus*	3	Mazandaran, Nowshahr, Madan forest	36˚42'393"	51˚23'92"	113	IAUH-000014978
C. schuschaensis	4	Mazandaran, Siah Bisheh	36˚12'393"	51˚35'92"	2017	IAUH-000014985
C. betulus var. *betulus*	5	Mazandaran, Siahbisheh	36˚62'393"	51˚25'92"	1759	IAUH-000014979
C. orientalis	6	Mazandaran, Chalus, Hezar Cham	36˚72'313"	51˚15'92"	1376	IAUH-000014986
C. schuschaensis	7	Mazandaran, Chalus, between Dordbon and Delir	36˚52'395"	51˚25'42"	1150	IAUH-000014987
C. orientalis	8	Mazandaran, Kojur, Dasht Nazir	36˚22'393"	51˚65'52"	963	IAUH-000014988
C. macrocarpa	9	Mazandaran, Kojour, Otaghsara village	36˚62'393"	51˚75'12"	1647	IAUH-000014989
C. betulus var. *betulus*	10	Gilan, Hashtpar	39˚52'393"	50˚35'92"	1730	IAUH-000014980
C. betulus var. *betulus*	11	Golestan, Golestan National Park	37˚42'393"	55˚ 75' 92"	785	IAUH-000014981
C. schuschaensis	12	Golestan, Gorgan, Radkan road	36˚92'391"	59˚15'22"	1205	IAUH-000014990
C. betulus var. *betulus*	13	Golestan, Gorgan Ziarat road	36˚51'51"	54˚02'28"	931	IAUH-000014982
C. betulus var. *betulus*	14	Golestan, Gorgan, Zarrin Gol	36˚52'393"	54˚23'92"	215	IAUH-000014983
C. orientalis	15	Golestan, 22 km Azad Shahr to Shahroud	36˚12'391"	55˚35'32"	479	IAUH-000014991
C. schuschaensis	16	Golestan,30km Azad Shahr to Shahroud	36˚52'392"	55˚25'92"	601	IAUH-0000149922
C. betulus var. *parva*	17	East Azerbaijan, Kaleybar to Ghaleh Babak	38°01'11"	47°23'26"	1096	IAUH-000014984

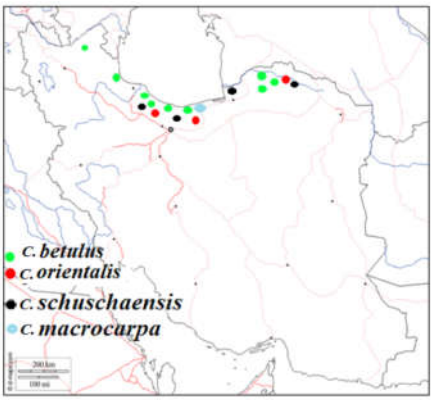

Fig. 1. Distribution map of the *Carpinus* populations studied.

Plant specimens were identified according to different references such as Flora Iranica (Browicz, 1972) and Flora U.S.S.R (Kuzeneva, 1936). Herbarium specimens are deposited in the herbarium of Science and Research Branch, Islamic Azad University (IAUH), Tehran.

Morphological data

All 85 collected individuals were used for morphological study and 36 morphological characters were measured for each individual (Table 3). Most of the evaluated traits are those used in previous studies (Browicz, 1972; Sabety, 2001; Chapolagh *et al.,* 2013; Adam *et al*., 2007; Razaz *et al*., 2013). Five individuals were examined per population and each character was measured randomly five times per individual and finally, the average of each feature was determined. Variables with deviation from normal distribution were log-transformed. We calculated Pearson and Spearman correlation coefficients to find variables with strong correlation (> 0.9) using IBM SPSS Statistics 26 (IBM Corp, 2019) software. Characteristics of leaves, stipule, petiole, fruit bract, and fruit were also studied on the plants. Finally, 36 characters (18 qualitative, 18 quantitative) were chosen for morphological analyses. Cluster analysis (CA) was performed for all characters and principal components analysis (PCA) was conducted based on quantitative characters using JMP11 software (1989-2007).

Table 3. Quantitative and Qualitative characteristics of the studied materials of *Carpinus*.

Abbreviation	Character	Abbreviation	Character
L_L	leaf length (cm)	NTCL	Number of teeth on central lobe of bract (absent= 0; 0.2-0.8=1)
L_W	leaf width (cm)	TML	Trichrome of leaf margin (absent= 0, present=1)
BW	distance from leaf base to the leaf maximum width (cm)	ULST	Upper leaf surface trichrome (absent=0, present=1)
L-S	stipule length(mm)	BL	Basal leaf (cordate= 0, rounded=1)
CLL	central lobe length of bracts (cm)	LS	Leaf shape (ovate= 0, elliptical= 1, oblong=2)
CLW	central lobe width of bracts (cm)	LA	Leaf apex (Acute= 0, Acuminate=1, caudato-Acuminate= 2)
LAL	leaf apex length (cm)	OL	Outer lobe of bract (convex=0, lobatae=1)
ILL	inner lobe length of bracts (cm)	ILM	Margins on the inner lobe of bract (whitout lobe= 0, Entire= 1, Etire-Erose= 2, Erose= 3)
ILW	inner lobe width of bracts (cm)	CLM	Margins on the central lobe of bract (Entire= 0, Erose Entire= 1, Erose= 2)
PL	petiole length (cm)	OLM	Margins on the outer lobe of bract (whitout lobe=0, Entire= 1, Etire-Erose= 2, Erose= 3)
V	number of leaf veins	FC	Fruit color (green= 0, brown= 2, black= 3)
NLF	number of lines on the fruit	FS	Fruit shape (late ovate= 0, ovate= 1, elliptic= 2)
SW	fruit width (cm)	FA	Fruit apex (truncatae = 0, acute= 1, apiculatus = 2)
SL1	fruit length (cm)	ATIL	top angle of inner lob of bract
IL	inner lobe of bracts (non lobatae=0, indistincte=1, lobatae= 2)	ABCIL	Angle between central and inner lobes on bract
DTOBCL	distance between top and outer base of central lobe (absent= 0; 0.74-1.08=1; 2.14-2.73=2)	ABCOL	Angle between central and outer lobes on bract
NTIL	number of teeth of inner lobe of bracts (absent= 0; present= 1)	FW	Fruit width (cm)
NTOL	number of teeth on outer lobe of bracts (absent= 0; 1-3= 1)	FL1	Fruit length (cm)

Molecular study

In total, 34 individuals from 17 collected populations of the genus *Carpinus* (two individual per each population) were sequenced for the present study. Moreover, we added 25 - GenBank sequences of the genus for the molecular study. *Betulus* and *Alnus* were selected as outgroups according to Yoo and Wen (2002) and Chapolagh *et al.* (2012). The list of non-Iranian taxa used in our analysis along with the GenBank accession numbers is indicated in table 4. Fresh leaves were taken randomly from each studied population and stored in silica gel. Genome extraction was performed using Nucleospin plant II extraction kits. PCR amplification was done for the entire ribosomal ITS region (ITS1+ 5.8S + ITS2) using primer ITS-AB101 and ITS -ab102 (Douzery *et al.*, 1999). The quality of PCR products was tested by being placed on 0.1 agarose gel.

Sequences were subjected to an automated alignment procedure using Clustal W (Thompson *et al*., 1994) under BioEdit v7.0.5.3 (Hall, 1999), and the resulting multiple alignments were corrected manually. Gaps were coded as informative sites in FastGap v1.2 (Borchsenius, 2009) according to the simple gap-coding method of Simmons and Ochoterena (2000). The sequence alignments used in the analyses are found in Online Resources 1- 2 (ESM 1- 2). Statistics on variation and information content of datasets were calculated with MEGA v7.0.21 (Kumar *et al*., 2016). For the determination of the taxonomic status of *Carpinus* species in Iran, phylogenetic trees were constructed based on maximum likelihood (ML) and Bayesian inference (BI) analyses and using data sets (ITS1+ 5.8S + ITS2). The TIM1+I+G best-fit of sequence evolution model for 10-million generations was selected in jModelTest v2.1.10 (Guindon and Gascuel, 2003; Darriba *et al*., 2012) under the Akaike

Information Criterion (AIC). ML analysis was carried out in raxmlGUI v1.5 (Silvestro and Michalak, 2012) the graphical interface of RAxML v7.2.6 (Stamatakis 2006), with 1000 replicates of bootstrap heuristic searches for nodal support (Felsenstein, 1985). Two parallel runs were done for 10-million generations, with 4 Markov chains and a sampling frequency of 1000 generations. The examination of the convergence of runs with accepting only the effective sample sizes > 200 for all parameters was conducted using Tracer v1.6 (Rambaut *et al*., 2014). The first 10% of the sampled trees were discarded as burn-in, and the remaining trees were used to construct a 50 percent majority-rule consensus tree visualized using FigTree.

Results

Morphological analysis

In the results of cluster analysis, the studied populations were grouped in two major clusters (Fig. 2). All populations of *C. btulus* placed in the first major cluster (cluster I) and populations of *C. orientalis*, *C. macrocarpa,* and *C. schuschaensis* fell into the second major cluster (cluster II). *C. betulus* var. *parva* are in cluster Ia and Cluster II was also divided into 2 sub-clusters. Sub-cluster IIa included populations of *C. orientalis*, Whilst *C. macrocarpa,* and *C. schuschaensis* were located in sub-cluster IIb (Fig. 2).

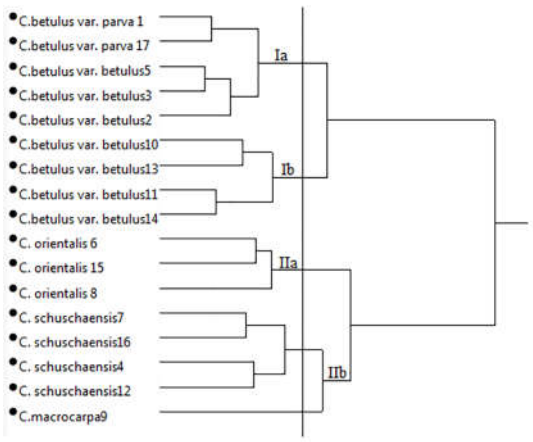

Fig. 2. Cluster analysis of *Carpinus* species based on quantitative and qualitative morphological data.

The PCA plot performed on quantitative morphological data revealed three groups. The two first principal components accounted for 69.8% of the total variance, 58% and 11.8% for the first and the second axes, respectively. Individuals of *C. betulus* located in the lower right and *C. orientals* differentiated very well in the left parts of the diagram (Fig. 3). Individuals of *C. macrocapa* and *C. schuschaensis* separated from the other taxa along the second axis. CLW, FW, and L_S have the most contribution in the separation of the studied taxa (Table 5).

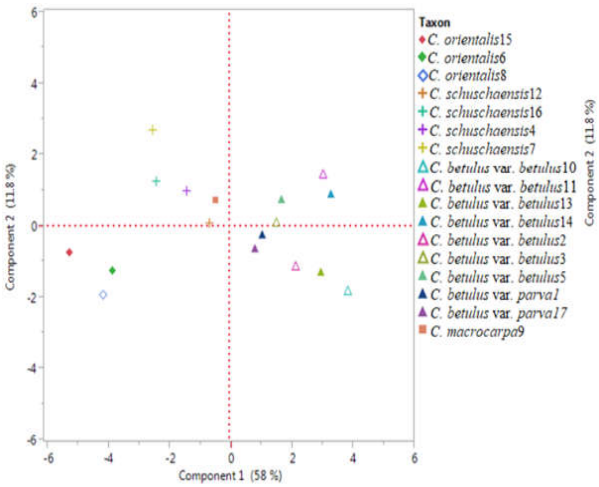

Fig. 3. Principal component analysis of the studied *Carpinus* taxa based on the quantitative morphological variables

Molecular analysis

The final alignment of the ITS data matrix consisted of 612 base pairs and 47 ITS sequences of the genus *Carpinus* (including 30 sequences from NCBI and 17 sequences from this study) among which 133 positions were phylogenetically variable and 65 were informative.

The overall topology of ML and BI analyses were similar. Therefore, we only presented the results of the Bayesian tree in Fig. 4. The results of the molecular analysis showed that the genus *Carpinus* is a monophyletic group. In the strict consensus tree, *Carpinus* consisted of two major clades. Clade I included species of the sect. *Distegocarpus* (*C. rankanensis* Hayata, *C. japonica* Bl and *C. fangiana* Hu) and all species of the sect. *Carpinus* is located in clade II. Clade II is represented by five sub-clades. Sub-

clade A contains C. betulus (PP= 100%, ML= 99 %). *C. tientaiensis*, *C. langaoensis*, *C. mianningensis* and *C. tschonoskii* formed sub-clade B (PP= 76%, ML= 79%). *C. macrocarpa*, *C. orientalis,* and *C. schuschaensis* specimens from Iran were placed in sub-clade C with strong support (PP= 100%, ML= 86%). *C. pubscens*, *C. kawakami*, *C. rupestris*, *C. mollicoma,* and *C. turczaninovii* are located in sub-clade D (PP= 100%, ML= 62%). Sub-clade E (clade E; PP= 100%, ML= 96%) included specimens of *C. londoniana* and *C. fargesiana*, *C. laxiflora* and *C. viminea*.) (Fig. 4).

Table 4. **Species** with clade affiliation, location, and GenBank accession number.

Species	**Locality**	**AcN***
C. betulus var. *parva* (clade A)	Iran, Mazandaran, Chamestan toWard waz forest	MN792640
C. betulus var. *betulus* (clade A)	Iran, Mazandaran, Kheyroud-Kenar forest	MN795490
C. betulus var. *betulus* (clade A)	Iran, Mazandaran, Nowshahr, Madan Forest	MN792798
Carpinus schuschaensis (clade C)	Iran, Mazandaran, Siah Bisheh	MN795134
C. betulus var. *betulus* (clade A)	Iran, Mazandaran, Siahbisheh	MN808611
Carpinus orientalis (clade C)	Iran, Mazandaran, Chalus, Hezar Cham	MN795483
Carpinus schuschaensis (clade C)	Iran, Mazandaran, Chalus, between Dozdbon and Delir	MN808610
Carpinus orientalis (clade C)	Iran, Mazandaran, Kojur, Dasht Nazir	MN795485
Carpinus macrocarpa (clade C)	Iran, Mazandaran, Kojour, Otaghsara village	MN795486
C. betulus var. *betulus* (clade A)	Iran, Gilan, Hashtpar	MN808612
C. betulus var. *betulus* (clade A)	Iran, Golestan, Golestan National Park	MN795478
Carpinus schuschaensis (clade C)	Iran, Golestan, Gorgan, Radkan road	MN795489
C. betulus var. *betulus* (clade A)	Iran, Golestan, Gorgan Ziarat road	MN808607
C. betulus var. *betulus* (clade A)	Iran, Golestan, Gorgan, Zarrin Gol	MN808606
Carpinus orientalis (clade C)	Iran, Golestan, 22 km Azad Shahr to Shahroud	MN808605
Carpinus schuschaensis (clade C)	Iran, Golestan,30km Azad Shahr to Iran, Shahroud	MN808609
C. betulus var. *parva* (clade A)	Iran, East Azerbaijan, Kaleybar to Ghaleh Babak	MN808604
Carpinus_betulus (clade A)	GenBank	AF432027
Carpinus tientaiensis (clade B_1)	GenBank	*JF796534*
Carpinus tientaiensis (clade B_1)	GenBank	*KX946976*
Carpinus_pubescens (clade D_1)	GenBank	*AF432050*
Carpinus langaoensis (clade B2)	GenBank	*KX946973*
Carpinus langaoensis (clade B_2)	GenBank	*KX946974*
Carpinus mianningensis (clade B_2)	GenBank	*KX946971*
Carpinus mianningensis (clade B_2)	GenBank	*KX946972*
Carpinus rupestris (clade D_2)	GenBank	*MG727562*
Carpinus mollicoma (clade D_2)	GenBank	*KX946977*
Carpinus londoniana (clade E_1)	GenBank	*JF796532*
Carpinus rankanensis (clade G_1)	GenBank	*FJ011727*
Carpinus fargesiana (clade E_1)	GenBank	*MG923676*
Carpinus tschonoski (clade B_2)	GenBank	*MH710986*
Carpinus tschonoskii (clade B_2)	GenBank	*FJ011733*
Carpinus_fangiana (clade G_1)	GenBank	*AJ783633*
Carpinus fangiana (clade G_1)	GenBank	*AF432034*
Carpinus turczaninovii (clade D_3)	GenBank	*JF831033*
Carpinus turczaninovii (clade D_3)	GenBank	*AF432056*
Carpinus japonica (clade G_2)	GenBank	*AJ783635*
Carpinus laxiflora (clade E_2)	GenBank	*AF432037*
Carpinus laxiflora (clade E_2)	GenBank	*AF432039*
Carpinus viminea (clade E_3)	GenBank	*AF432058*
Carpinus kawakamii (clade D_1)	GenBank	*FJ011720*
Carpinus viminea (clade E_3)	GenBank	*AF432058*
Betula glandulosa	GenBank	*KT309017*
Ostryo carpinifolia	GenBank	*AF432059*
Ostryopsis nobilis	GenBank	*KC412171*
Corylus heterophylla	GenBank	*AF297352*
Alnus accuminata	GenBank	*AJ251673*

*AcN= Accession numbers

Table 5. Details of quantitative and qualitative morphological characters were used in the present study.

Character*	Principal 1	Principal 2
L_L	0.3406489	-0.077157
L_W	0.3046627	-0.057929
BW	0.3269439	-0.11286
LAL	0.2883221	0.0158798
PL	0.3180824	0.0911841
FL1	0.2451404	0.048352
CLL	0.3171658	0.0474144
ILL	0.3198263	0.1402485
ILW	0.3030707	0.1731279
NLF	0.2484672	-0.032397
L_S	-0.03519	0.525151
FW	0.1198701	0.546273
V	0.2083597	-0.130798
CLW	-0.156061	0.568156

*L_L= leaf length (cm); L_W= leaf width (cm); BW= distance from leaf base to the leaf maximum width (cm); LAL= leaf apex length (cm); PL= petiole length (cm); FL1= fruit length (cm); CLL =central lobe length of bracts; ILL= inner lobe length of bracts (cm); ILW= inner lobe width of bracts (cm); NLF; number of lines on the fruit; L_S= leaf shape; FW= Fruit width (cm); V=number of leaf veins; CLW=central lobe width of bracts (cm).

Discussion

The result of ITS tree showed that Iranian species of the genus *Carpinus* were located in two different well-supported clades. *C. betulus* was closer to *C. tschonoskii* and *C. orientalis*, *C. macrocarpa*, *C. schuschaensis* showed a sister-group relationship with *C. pubscens*, *C. kawakami, C. rupestris, C. mollicoma,* and *C. turczaninovii* (Fig. 4). These relationships were also reported by Yoo and Wen (2002) and Sun *et al*. (2011). The clear separation of *C. betulus* and *C. orientalis* in the ITS tree is also supported by other lines of evidence. Morphologically, these species belong to different subsections of sect. *Carpinus* (Yoo and Wen, 2002). Furthermore, each species has a different ploidy level with 2n= 8x= 64 for *C. betulus* and *C. orientalis* in 2n= 2x= 16. In addition, Carabus *et al*. (2017) investigated the genetic diversity of *C. betulus* and *C. orientalis* in Romania and the results of their study showed that these two species do not share common chloroplast haplotypes, even when they occur in sympatric areas.

Taxonomic histories of *C. macrocapa* and *C. schuschaensis* have been controversial. *C. macrocapa* was firstly described by Willkomm (1887) as a variety of *C. oreintalis*, *C. orientalis* var. *macrocarpa*. Later, Winkler (1904) suggested that this taxon differs from *C. orientalis* by having larger leaves and fruit bracts and elevated *C. macrocapa* to species level. Browicz (1972), considering the differences in leaf and fruit bract size, divided *C. orientalis* into two subspecies, *C. orientalis* subsp. *macrocarpa* with larger leaves and fruit bracts and *C. orientalis* subsp. *orientalis* with the smaller ones. However, he emphasized it is possible that subsp. *macrocarpa* is only a hybrid form closely resembling *C. orientalis*. *C. schuschaensis* was firstly identified by Winkler (1904). According to Browicz (1972) *C. schuschaensis* with variable bract size and shape, intermediate leaf and height, represents a hybrid form of *C. betulus* and *C. orinetalis* because hybridization appears to be common in sympatric areas of the above-mentioned species. Therefore, he recognized *C. schuschaensis* as a hybrid of *C. betulus* and *C. orinetalis*. This view was also adopted by Ghahreman (2000). On the other hand, Chapolagh Paridari *et al.,* (2012) revised the taxonomy of the genus *Carpinus* in Iran using molecular data (nrDNA ITS and *trnH-psbA*) and the results of their study did not confirm the species status of *C. schuschaensis*. They concluded that this taxon lies within the intra-specific morphological variation of *C. orientalis* and synonymized *C. schuschaensis* under the former taxon.

In the present study, although *C. macrocapa* and *C. schuschaensis* showed an intermediate position between *C. betulus* and *C. orientalis,* in the PCA plot of morphological data, ITS sequences of *C. macrocapa* and *C. schuschaensis* produced clean chromatograms identical in sequence to *C. orientalis* and these three species formed a monophyletic group with high support in the ITS tree (Figs. 3 and 4).

Moreover, in the cluster analysis of morphological data, *C. macrocapa*, *C. schuschaensis,* and *C. orientalis* were included in the main cluster with two sub-clusters (Fig. 2). Based on the morphological differentiation of *C. macrocapa*, we agree with those who consider this taxon as a distinct subspecific taxon of *C. orientalis* (Browicz, 1972; Mozaffarian, 2005). Razaz *et al.,* (2013) investigated the morphological diversity of *C. orientalis* in 32 populations and stated that subdividing this species into two subspecies based on two main diagnostic characters (leaf and bract size) is not valid because there is no correlation between these characters. In our morphological study, leaf size exhibited a continuous variation among

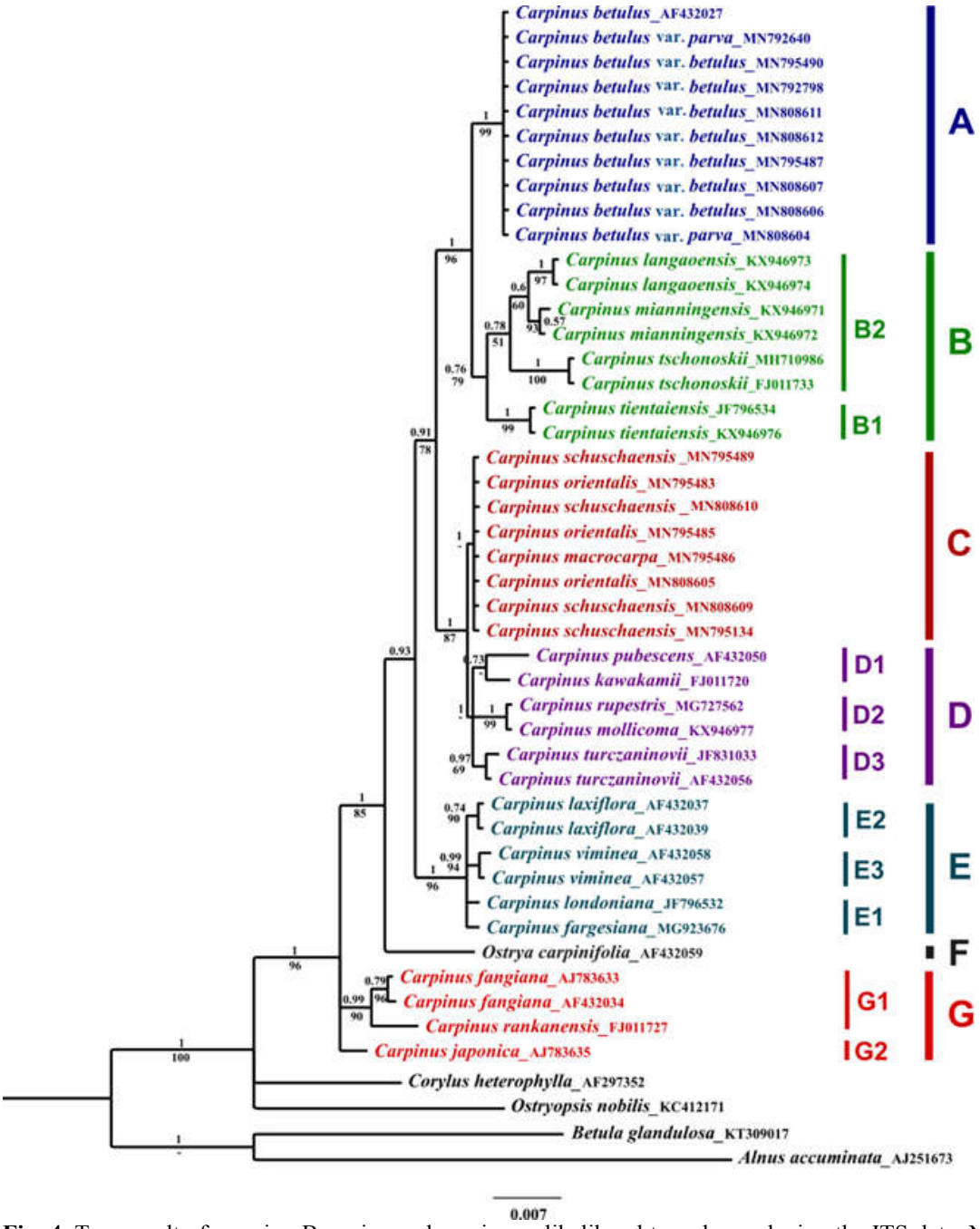

Fig. 4. Tree result of merging Bayesian and maximum likelihood trees by analyzing the ITS data. Numbers above the branches are posterior probabilities (PP) values of Bayesian inference (BI), and numbers below the branches are values of maximum likelihood (ML).

C. orientalis, *C. macrocapa,* and *C. schuschaensis*.

In contrast, morphological characters such as the width of central lobe bract and stipule length, as well as fruit width are useful in the separation of *C. orientalis* from *C. macrocapa* and *C. schuschaensis* (Table 4). Furthermore, these taxa show different geographical distributions in the world. Whereas *C. orientalis* is distributed from southeastern Europe to northern Iran, the distribution range of *C. macrocapa* and *C. schuschaensis* is restricted to the Hyrcanian forest of Iran, Talish in the Azerbaijan Republic, and Caucasus austro-orientalis (Browicz, 1972).

In the case of *C. schuschaensis*, we concur with Chapolagh *et al*. (2012). However, as subs. *macrocarpa* was published before *C. schuschaensis,* we suggest that this taxon should be treated as a synonym of *C. orientalis* subsp. *macrocarpa*.

The results of our study revealed that, compared to hybridization, intra-specific morphological diversity has caused more taxonomic confusion in the taxonomy of the genus *Carpinus* in Iran. The results of our morphological study is following Razaz *et al*. (2013) who observed high levels of variation in quantitative and qualitative morphological characters of *C. orientalis*. This morphological diversity could be explained by differences in environmental conditions such as elevation. This species grows in different elevation levels from 200 m to 2600 m (Browicz 1972; Razaz *et al*., 2013).

Based on the differences in the bract and leaf size, two varieties have been mentioned for *C. betulus* in flora Iranica and our study, showing an intraspecific division for this species.

Taxonomic treatment

Key to the taxa

1) Bracts 3- lobed; margin of lobes entire or rarely dentate. The number of stria of bract 3. Distance between central lobe apex from outer lobe base 2.14-2.73 cm. leaf length (5) 6-10 (12) × 2/8-6/3 cm.....1. C. betulus

- Bracts ovate or 2 (-3) lobed; both sides dentate. The number of stria of bract 4-7. Distance between central lobe apex from outer lobe base 0-1/08 cm. Leaf length 4/02-6cm.....2

2) Stipule length 0/2-0/28 cm. Central lobe width 0/67-0/9 cm. Seed width 0/32-0/41 cm. Bracts not lobed.....2. ***C. orientalis*** subsp. ***orientalis***

Stipule length 0/33-0/44 (0/24) cm. Central lobe width 0/92-1/4 cm. Seed width 0/46-0/62 cm. Bracts 2 (-3) lobed; outer lobe dentate (erose) **……*C. orientalis*** subsp. ***macrocarpa***.

C. betulus L., Spee. Plant. 998 (1753).

Syn.: C. caucasica GROSSH., IZV. Azerb.Fil. Akad. Nauk SSSR. 5:34 (1940).

Icon.: REICHENB., Icon. Fl. Germ. 12: tab. *632, 633 (1850).*

A tree of about 25 m in height. Deciduous; bark grey; trunk rounded-ribbed. Branchlets brown. Buds up to 10 mm long narrowly oblong. Covered with numerous scales; scales reddish-brown, ciliate., Leaf elliptical or oblong, (5) 6-10 (12) × 2/8-6/3 cm, at the base rounded or cordate, in upper surface glabrous or sparsely villous, in lower surface villous, acuminate or mucronate, 0/2-0/8 × 0/3-0/9 cm, at the margin irregularly double serrate; number of leaf veins 11-16; petiole length 0/9-1/5 cm; stiuple length 0/4-0/7 cm; fruit bracts 3 lobed, central lobe 2/97-3/66 × 0/6– 0/92 cm, without teeth or rarely dentate, inner lobe of bract 1/13-1/86 × 0/32-0/56 cm, distinct, without teeth, outer lobe 1-2 × 0/3-0/6 cm, at the margin entire; distance between central lobe apex from outer lobe base 2/14-2/73 cm; inflorescence 2-5 cm long. pendulous, in fruiting state up to nearly 15 cm long, 6 cm wide; nutlets 0/5-0/7× 0/38-0/56 cm, late ovate, ovate or rarely elliptic, brown or green, at the apex truncatae or acutish ; number of lines on the nutlet 12-13.

Distribution and habitat: Central Europe to South-east, Anatolia, Caucasus, Northern Iran.

--var ***betulus***

Type: Europa, LINN 1131.1.; Central lobe length of bracts 2/7-4× 0/7-1 cm.; Selected specimens seen: Iran, Mazandaran Province: Kheyroud-Kenar Forest, Riahee IAUH-000014977: Nowshahr, Madan Forest, Riahee IAUH-000014978. Gilan Province: Hashtpar, Riahee IAUH-000014980. Golestan Province: National Park, Riahee IAUH-000014981, Gorgan, Ziarat Road, Riahee IAUH-000014982; Zarrin Gol, Riahee IAUH-000014983.

--var ***parva*** RADDE-FOMIN, Mem. Cl. Sciene. Phys. et Math., Acad. Scienc. de l'Ukraine 15,1;81 (1929).

Type: Dagestan, Agan kale prope Temir-chan-Schura, 1916, BUDAEV

Central lobe length of bracts 1/8-2/2×0/3-0/5 cm. Leaves smaller than var. *betulus*.

Selected specimens: Iran, Mazandaran Province: 20 km from Chamestan toward Waz Forest, Riahee IAUH-000014976. East Azerbaijan Province: Kaleybar to Ghaleh Babak, Riahee IAUH-000014984.

2. ***C. orientalis*** MILLER, Gard. Dict. ed. 8 no.3 (1768)
Syn.: C. duinensis Scop., Fl., Carn. ed. 2; 243, tab.60 (1772).
Icon: C. K. SCHNEIDER, I11. Handb. Laubholzk, I, tab. 78 (1904); RADDE-FOMIN; Mem. Cl. Science. Phys. et Math., Acad. Scienc. de I'Ukraine 15, 1: tab. 7a (1929*)*.
Robust Shrub or small tree to nearly to 4-5 (-8) m high. Young branches and petioles silky- hairy. Leaves oval, oval-ellipsoidal or ellipsoidal, 4/2-6×2/34-3/7 cm, at the base cordate or rarely rounded; both surfaces villous, densely villous along the veins, at the apex acute or acuminate 0-0/6×0-0/4cm, at the margin irregularly double serrate; the number of leaf veins 10-14; petiole length 0/6-1 cm; stipule length 0/2-0/44 cm. Inflorescence dense, in fruiting state 3-6 (-8) cm long, 2-3/5 cm board. Fruit bracts triangular-ovate or 2 (3) lobed; central lobe 1/82-4×0/67-1/14 cm; inner lobe 0-0/8×0-1/15 cm; outer lobe of bract 0-2/2×0-/7cm; non-lobed bracts entire, erose-entire or erose at the margin with 1-5 teeth; distance between central lobe apex from outer lobe base 0-1/08 cm. Nutlets 0/4-0/9×0/32–0/62, late ovate, ovate or elliptic, green, black or rarely brown, at the apex truncatae or apiculate; the number of lines on the fruit 8 - 12.
Distribution and habitat: South-eastern Europe, Anatolia, northern Syria, Caucasus and N., and NW Iran.
--subsp***. orientalis***
Type: Southern Europe
Stipule length 0/2-0/28 cm. Bracts not lobed Seed width 0/32-0/41 cm.
Selected specimens: Iran: Mazandaran Province: Chalus, Hezar Cham, Riahee MN795483: Kojur, Dasht Nazir, Riahee MN795485. Golestan Province, 22 km from Azad Shahr toward Shahroud, Riahee MN808605.
--subsp. ***macrocarpa*** (WILLk) BROWIZ, F*lora* Iranica 97: 2 (1972).
Syn*.: C. orientalis* MILLER var. *macrocarpa* WILLK., Forstl. Flora 368 (1887*); C. macrocarpa* (WILLK.) WINKL. in ENGLER gR Pflanzenr. IV-61:38 (1904); *C. hybrida H.* WINKL. in ENGLER, Pflanzenr. IV-61: 40 40 (1904). *C. schuschaensis* WINKL in ENEGLER, Pflanzenr. IV-61: 32 (1904); *C. grosseserata H.* WINKL., l. c. *C. geokezaica* RADDE-Fomin. Mém.CI. Sciene. Phys. et. Math., Acad. Sciene. de Í'Ukraine 15, 1:89 (1929*)*.
Typus: Turcomania, Hohenacker.
Stipule length 0/33-0/44 (0/24) cm. Central lobe width 0/92-1/4 cm. Seed width 0/46-0/62 cm. Bracts 2 (-3) lobed, outer lobe dentate (erose).
Selected specimens: Iran, Mazandaran Province: Siah Bisheh, Riahee MN795134; Chalus Valley, 6 km from Dordbon to Delir, Riahee MN795485. Golestan Province: 22 km from Azad Shahr to Shahroud. Riahee MN808609.

Conflict of interests

The authors declare that they have no conflict of interest.

References

Abdi E, Hassan R, Baris M. 2009. Distribution and tensile strength of Hornbeam (*Carpinus betulus*) roots growing on slopes of Caspian Forests, Iran. *J Forest Res* 20(2): 105-110.

Alvarez I, Wendel JF. 2003. Ribosomal ITS sequences and plant phylogenetic inference. *Mol Phyl Evol* 29: 417-434.

Bergmeier E, Dimopoulos P. 2008. Identifying plant communities of thermophilous deciduous forest in Greece: species composition, distribution, ecology, and syntaxonomy. *Plant Biosyst* 142:228-254.

Boratyński A, Boratyńska K, Mazur M, Marcysiak K. 2007. Seed involucre variation in *Carpinus betul* (Corylaceae) in Poland. *Act Biol Cracovies* 49(1): 103-111.

Borchsenius F. 2009. FastGap 1.2. Department of Biological Sciences, University of Aarhus, Aarhus. Available at: http://192.38.46.42/aubot/fb/FastGap_home.htm

Browicz K. 1972. Corylaceae. In: Rechinger, K. H. (ed.), Flora Iranica. Akad. Drucku. verlagsanstalt, Graz, 97: 91-100.

Carabus MC, Curtu AL, Postolache D, Ciocirlan E, Şofletea N. 2017. Evidence of low chloroplast genetic diversity in two *Carpinus* species in the northern Balkans. *Notulae*

Botanicae Horti Agrobotanici Cluj-Napoca 45(1): 316-322.

Čarni A, Košir P, Karadžić B, Matevski V, Redžić S, Škvorc Ž. 2009. Thermophilous *Carpinus* species using of molecular markers (nrDNA ITS and trnH-psbA). *Iran J Rangelands Forests Plant Breed Genet Res* 20(1): 1-13 (in Persian).

Colagar AH, Razaz M, Naqinezhad A, Mahmoudi Otaghvari A. 2015. Intraspecific study of *Carpinus orientalis* populations using seed storage protein pattern. *Appl Biol* 28(2):33-47.

Douzery E, Pridgeon A, Kores P, Linder HP, Kurzweil H. Chase M. 1999. Molecular phylogenetics of diseae (Orchidaceae): a contribution from nuclear ribosomal ITS sequences. *Am J Bot* 86(6): 887-899.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evol* 39(4): 783-791.

Fini A, Ferrini F. 2011. Effects of mulching with compost on growth and physiology of Acer campestre L. and Carpinus betulus L. *Adv Hortic Sci* 25(4): 232-238.

Ghahreman A. 2000. Biodiversity of plant species in Iran, vol. 1. Tehran University Publications. Tehran. Iran.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 52: 696-704.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* 41: 95-98.

Holstein N, Weigend M. 2017. No taxon left behind? a critical taxonomic checklist of *Carpinus* and Ostrya (Coryloideae, Betulaceae). *Eur J Tax* 375: 1-52.

IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.

Jeon JI, Chang CS, Chen ZD, Park TY. 2007. Systematic aspects of foliar flavonoids in subsect. *Carpinus* (*Carpinus*, Betulaceae). *Biochem Syst Ecol* 35(9): 606- 613.

JMP®, Version 11. SAS Institute Inc., Cary, NC, 1989-2019.

Kress W J, Wurdack K J, Zimmer EA, Weigt LA, Janzen DH. 2005. Use of DNA barcodes to deciduous forests in Southeastern Europe. *Plant Biosys* 143(1): 1-13.

Chapolagh Paridari I, Jalali Gh, Sonboli A, Zarafshar M. 2012. Revision of Iranian identify flowering plants. *Proc Natl Acad Sci USA*, 102(23): 8369-8374.

Kress WJ, Erickson DL. 2007. A two-locus global DNA barcode for land plants: the coding rbcL gene complements the non-coding *trnH- psbA* spacer region. *PLoS One PLoS One* 2(6), e508.

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

Kuzeneva OI. 1936. Betulaceae. In: Komarov VL, editor Flora of the U.S.S.R. vol. 5. Moskva & Leningrad. 269-319.

Li Y, Yang YZ, Yu L, Du X, Ren GP. 2018. Plastomes of nine hornbeams and phylogenetic implications. *Ecol Evol* 8(17): 8770-8778.

Linnaeus C, Stearn WT. 1960. Genera plantarum (pp. 1789-91). Weinheim, Engelmann.

Miller MA, Pfeiffer W, Schwartz T. 2010. Creating the CIPRES science gateway for inference of large phylogenetic trees. In: Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA, pp 1-8.

Mozaffarian V. 2005. Trees and shrubs of Iran. Farhang-e Moaser Publication, Tehran pp, 205-209.

Rambaut A, Suchard MA, Xie D, Drummond AJ. 2014. Tracer v1.6. Available at: http://beast.bio.ed.ac.uk/Tracer.

Razaz M, Naqinezhad A, Mahmoudi Otaghvari A, Colagar AH, Abbas Azimi R. 2015. Comparative anatomy of *Carpinus orientalis* Mill. (Betulaceae) populations in Iran. *J Genet Resour* 1(1): 45-54.

Razaz M, Naqinezhad A, Mahmoudi A, Hosseinzadeh A, Zare H. 2013. A study of *Carpinus orientalis* Mill. populations based on morphological characteristics in the hyrcanian forests, N. Iran. *Adv Biores* 4 (3): 98-104.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572-1574.

Sabety H. 2001. Tree and shrubs of Iran. Yazd University, 791pp.

Silvestro D, Michalak I. 2012. raxmlGUI: a graphical front-end for RAxML. *Organ Divers Evol* 12(4): 335-337.

Simmons MP, Ochoterena H (2000) Gaps as characters in sequencebased phylogenetic analyses. *Syst Biol* 49:369-381.

Small R L, Cronn RC, Wendel JF. 2004. LAS Johnson Review No. 2. Use of nuclear genes for phylogeny reconstruction in plants. *Aust Syst Bot* 17: 145-170.

Soltis DE, Soltis PS, Pires JC, Kovarik A, Tate JA, Mavrodiev E. 2004. Recent and recurrent polyploidy in *Tragopogon* (Asteraceae): cytogenetic, genomic and genetic comparisons. *Biol J Linn Soci* 82:485-501.

Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2688- 2690.

Sun YL, Wang D, Kim YS, Park WG, Kwon OW, Hong SK. 2011. Phylogeny and evolution of *Carpinus* based on nrDNA ITS region sequences, International Conference on Biomedical Engineering and Informatics (BMEI).

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl Acids Res* 22: 4673- 4680.

WCSP. 2016. World Checklist of Selected Plant Families. Facilitated by the Royal Botanic Gardens, Kew. Available from: http://apps.kew.org/wcsp/ [accessed 8 Jul. 2016

Willkomm H.M. 1887. Forstliche Flora von Deutschland und Oesterreich ed. 2. C.F. Winter'sche Verlagshandlung, Leipzig and Heidelberg.

Winkler H. 1904. Betulaceae. In: Engler, A. (Ed.), Das Pflanzenreich, IV, 61. W. Engelmann, Leipzig, pp. 1e149.

Yoo KO, Wen J. 2002. Phylogeny and biogeography of *Carpinus* and subfamily Coryloideae (Betulaceae). *Int J Plant Sci* 163: 641-650.

Yoo KO. Wen J. 2007. Phylogeny of *Carpinus* and subfamily Coryloideae (Betulaceae) based on chloroplast and nuclear ribosomal sequence data. *Plant Syst Evol* 267: 25-35.

Yousefzadeh H, Colagar AH, Tabari M, Sattarian A, Assadi M. 2012. Utility of ITS region sequence and structure for molecular identification of *Tilia* species from Hyrcanian forests, Iran. *Plant Syst Evol* 298: 947-961.

Yousefzadeh H. Colagar AH, Yousefi E, Badbar M, Kozlowski G. 2019. Phylogenetic relationship and genetic differentiation of *Populus caspica* and *Populus alba* using cpDNA and ITS noncoding sequences *J Forest Res* 30(2):451-461.

Zhang CY, Wang FY, Yan HF, Hao G, Hu CM, Ge XJ. 2012. Testing DNA barcoding in closely related groups of *Lysimachia* L. (Myrsinaceae). *Mol Ecol Res*12(1): 98-108.