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Phylogenetic relationships of *Pterocarya* (Juglandaceae) with an emphasis on the taxonomic status of Iranian populations using ITS and *trn*H-*psb*A sequence data

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Abstract

Pterocarya fraxinifolia (Lam.) Spach., a relict tree species of the Juglandaceae family, is native to the Great Caucasus, Anatolia, and to the Hyrcanian forests of the southern Azerbaijan and Northern Iran. In this study, the phylogenetic relationship of the species, sampled in selected Iranian populations, and the global biogeography of the genus *Pterocarya* were addressed. Leaves were collected from 8 to 10 trees from three geographically isolated habitats. The samples were analyzed with nuclear (internal transcribed spacer [ITS] regions) and chloroplast (*trn*H-*psb*A) DNA markers. The obtained results were compared and analyzed with the data registered in NCBI GenBank. It is reported that the ITS regions varied from 644 to 652 for *Pterocarya* genus, but we did not observe polymorphisms for Iranian *Pterocarya*. The phylogenetic tree divided the *Pterocarya* genus in three clades: clade 1 grouping exclusively the samples *P. fraxinifolia*, clearly separated from the East Asiatic taxa; clade 2 that includes the species *P. hupehensis* and *P. macroptera*; clade 3 clustering *P. stenoptera* and *P. tonkinensis*. Although the Iranian *Pterocarya* samples and *P. fraxinifolia* from the Caucasus were in the same clade, they presented two different secondary structures. The Iranian populations showed the maximum genetic distance with *P. stenoptera* and *P. tonkinensis*. Our analysis demonstrates that the traditional division of all the six species sampled throughout their distribution area as well as the phylogeny of the genus *Pterocarya* needs to be reviewed.

Keywords: *Biogeography, DNA barcoding, molecular identification, phylogeny, Hyrcanian forests*

Introduction

Hyrcanian forests cover the northern slope of the Alborz Mountains in Iran and the Talysh Mountains in southern Azerbaijan. In Iran, these forests (1.84 million hectares) corresponding to 15% of the total forest area, stretching from the plains up to a height of 2500 m a.s.l. (exceptionally 2800 m) (Sabeti 1994; Sagheb-Talebi et al. 2004; Khosroshahi & Ghavvami 2006). In total, 3234 species belonging to 856 genera and 148 families of vascular plants have been reported that in the Hycranian forest of Iran.

Several emblematic relict tree species, including *Gleditsia caspica*, *Zelkova carpinifolia*, and *Pterocarya fraxinifolia*, are indicators of these forests (Akhani et al. 2010). Currently, there are six extant species of the genus *Pterocarya*, with *P. fraxinifolia* growing in western Eurasia (Transcaucasia, Anatolia and the Hyrcanian region) and five other species in eastern Asia (mainly in China, the Koreas and Japan) (Rix 2007). In the past, however, *Pterocarya* genus possessed a much broader distribution in the whole northern hemisphere, with fossils dating to the early Oligocene (32 Ma) (Manchester 1987, 1989; Manos et al. 2007).

Pterocarya fraxinifolia is a light-demanding, thermophilous tree with a maximum height of 35 meters that is naturally distributed throughout the western Black Sea region of Turkey and is native to the Caucasus from Northern Iran to Ukraine (Kayacik 1981; Davis 1982; Ansin et al. 1998; Nabavi et al. 2008). The species has deeply fissured bark, pinnate leaves with 5–12 leaflets (with strongly serrated edges with 50 to 65 teeth on each side) and winged nut fruits. Leaves and bark of *P. fraxinifolia* contain juglone; native people use the leaf of this tree as an anesthetic agent to catch fish, for dyeing and as an antifungal agent (Hadjmohammadi & Kamel 2006). The flowers are monoecious and appear in

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April. The male catkins are thick and green, 7.5–12.5 cm long; the females, green and winged, and have less dense flowers, and bear red styles that form fruiting catkins 30–50 cm long.

The seeds have a small edible nuts about 5–10 mm of diameter, with two wings, one each side. Although the wood of *P. fraxinifolia* is little employed in carpentry (Leroy et al. 2013), the tree is widely used as a landscape element in European gardens and parks (Gungor et al. 2007). The tree grows best in flat sites or slopes with low gradients, at an altitude of 400–700 m above sea level and near river banks on semi-deep and moist soils. Climatic conditions associated with the species distribution are generally mild winters and summers and very humid regimes (Shekholislami & Ahamadi 2009). The average annual rainfall and temperature in Hyrcanian forests with *P. fraxinifolia* are 1386 mm and 14.7°C, respectively. *P. fraxinifolia* is a fast-growing tree species that prefers to live and grows in mixture with other species while seldom forms pure stands. In the Hyrcanian forest, the species is accompanied by *Alnus glutinosa*, *A. subcordata*, *Acer velutinum*, *Carpinus betulus*, and *Populus caspica*.

Based on pollen studies and the reconstruction of vegetation history, Ramezani (2013) stated that *P. fraxinifolia* had a high frequency in the past, especially about 700 years ago. The decreasing of *Pterocarya* species in Hyrcanian forests coincides with the climatic fluctuation during the middle ages. At that time, due to the drier conditions and warming (apparently a trans-regional phenomenon), the frequency of *P. fraxinifolia* was reduced, or at least its distribution range was limited to lower elevations. Confirming the decline of the distribution of *P. fraxinifolia* in Iran around 780–1350, Leroy et al. (2013) stated that this species is sensitive to climate changes.

The taxonomy and phylogenetic relationships within the genus *Pterocarya* are not well understood. Molecular sequence data along with phylogenetic analyses are increasingly being used for species identification and evolutionary inference in many plant species. The internal transcribed spacer (ITS) regions of nuclear ribosomal DNA has been frequently used to determine phylogenetic relationships in plants (Potter et al. 2000; Fernández et al. 2001; Samuel et al. 2003; Fukuda et al. 2011; Yousefzadeh et al. 2012). Due to the small size (approximately 700 base pairs (bp)) and adequate variation in (the rate of nucleotide substitutions) universal primers as well as easy amplification, this region was selected for use in a phylogenetic analysis of plants at lower taxonomic levels. Additionally, the *trn*H-*psb*A region in the chloroplast genome is another suitable DNA marker for phylogenetic studies from the intra-species to the interfamily level (Lahaye et al. 2008; Newmaster et al. 2008; Yousefzadeh et al. 2014).

Using both nuclear and chloroplast DNA markers is advantageous in discerning hybrid species due to their different patterns of inheritance. For example, Ren et al. (2010) concluded that the combination of two regions, the ITS and *trn*H-*psb*A, is the best choice for DNA identification of the *Alnus* species, as an improvement and supplement for morphological-based taxonomy. In this study, we aim to assess the taxonomic status and degree of molecular relationship of selected Iranian *P. fraxinifolia* populations within the genus *Pterocarya* and, more generally, to elucidate the relationships of the genus *Pterocarya* within the Juglandaceae family.

Materials and methods

Sampling, DNA extraction, ITS, and trnH-psbA amplification and sequencing

Sampling of *P. fraxinifolia* was done in three geographically isolated populations in Iran (Figure 1 and Supplemental Material); for each population, leaves from 10/8 mature trees were collected. Selected trees were at least 20 m apart from each other to decrease the possibility of being the components of the same parent. Leaves were frozen in liquid nitrogen and milled to a fine powder. Total cellular DNA was extracted from the ground powder using an AccuPrep Genomic DNA Extraction Kit (Bioneer, Korea: www.Bioneer.com). The ITS regions were amplified on a thermal cycler (Bio-Rad) using the ITS1 forward primer: 5′-TCGAAACCTGCCTAGCAG-3′ and ITS4 reverse primer: 5′-ATGCGAGCATCGTTCGA-3′ (White et al. 1990). Forward primer *trn*H GUG (5′-CGC GCATGG TGG ATT CACAATCC-3′) (Tate & Simpson 2003) and the reverse primer *psb*A (5′-GTT ATG CAT GAA CGT AAT GCT C-3′) (Sang et al. 1997) were used to amplify the *trn*H-*psb*A region. DNA amplifications were carried out in a 25-μL reaction mixture containing master mix RED (Ampliqon-Denmark), 0.7 mM of each primer, and 20 ng of template DNA. The thermal cycling profile consisted of an initial denaturation step of 360 s at 95°C, followed by 32 cycles of 60 s at 95°C, 45 s at 56°C, 90°s at 72°C and a final extension step of 5–7 min at 72°C. Agarose electrophoresis (1%) was performed to visualize polymerase chain reaction (PCR) products after ethidium bromide staining.

Phylogenetic analysis

Electrophenograms of the ITS and *trn*H-*psb*A sequences were further visually analyzed using the Chromas software program version 2.4.0.0. The boundaries of the ITS1 and ITS2 regions were determined via BLAST search (http://blast.ncbi.

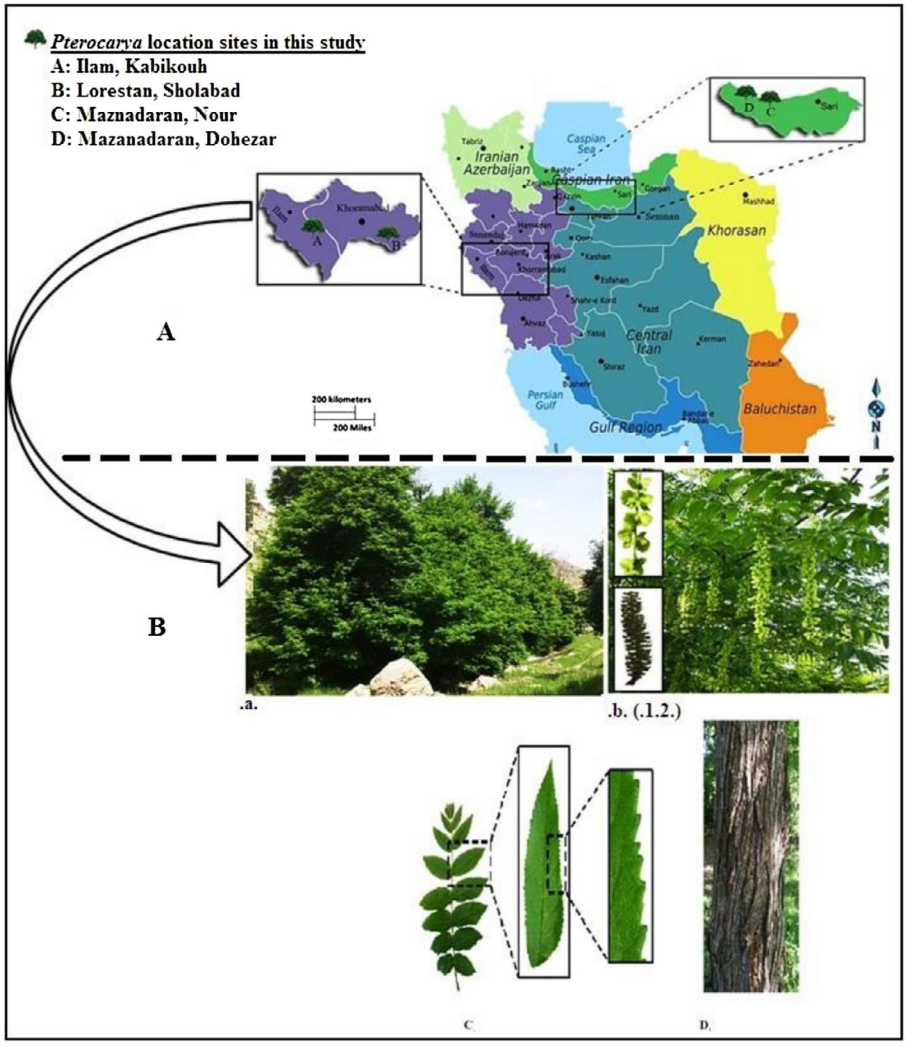

Figure 1. (A) Map of the Iranian areas (violet, Zagrosian region; green, Hyrcanian region) where *P. fraxinifolia* populations were sampled for this study (geographical coordinates in Supplemental data). (B) Typical habitat and morphology of *Pterocaria fraxinifolia* species: (a) Habitat of Ilam area; (b) flowers (1. female and 2. mail catkins); (c) leaf serration; (d) bark.

nlm.nih.gov/) and compared with known sequences of *Pterocarya* from NCBI GenBank (nucleotide collection nr/nt database). Phylogenetic trees were constructed using three data-sets, nrDNA (ITS1 + ITS2), and *trn*H-*psb*A. Due to the lack of polymorphisms in the *trn*H-*psb*A intergenic spacer region, this region was discarded from the analysis.

Based on the sequence alignment, we filtered all positions that were variable between (and within) main-genotype *Pterocarya* individuals throughout their distribution range. To determine appropriate models of the sequence evolution for all the data-sets, model tests in MEGA 5.2 (Tamura et al. 2011) and the Tamura 3-parameter model with the gamma-distributed rate heterogeneity parameter were selected. This model of nucleotide substitution and five gamma rate categories were employed to estimate the phylogeny using the maximum likelihood method. Relative support for individual clades was established using a bootstrap analysis, based on 1000 replicates; nodes with bootstrap values of 50% were considered to be well supported.

Furthermore, a phylogenetic network was constructed using the median-joining algorithm with the set of SNPs identified in the ITS and *trn*H-*psb*A intergenic spacer. For median-joining (MJ) network analysis, all variable characters of the complete alignment were entered into the PopART software (http://popart.otago.ac.nz).

The genera *Juglans*, *Carya*, and *Cyclocarya* were used as outgroups in the present study, and their sequences were selected from NCBI GenBank deposits.

Table I. Characteristics of the aligned ITS data matrix used for phylogenetic analyses.

	Iranian populations[a]								
	Hyrcanian *Pterocarya*			Zagrosian *Pterocarya*			All taxa		
	ITS1	5.8S	ITS2	ITS1	5.8S	ITS2	ITS1	5.8S	ITS2
A (%)	20.3	24.4	15.9	20.5	24.4	25.9	21.1	23.4	15.9
C (%)	28.3	26.2	31	28.3	26.2	31	28.3	27.2	30.5
G (%)	28.7	27.2	29.2	28.7	27.4	29.2	27.7	27.9	28.7
T (%)	22.6	22	23.9	22.4	22	23.9	22.9	21.6	24.9
Total length (bp)	255	164	226	255	164	226	270	178	228
Conserved sites	252	164	226	254	164	226	260	178	223
Variable sites	1	–	–	–	–	–	10	–	3
Parsimony informative sites identical pairs	–	–	–	–	–	–	8	–	2
Singleton	–	–	–	–	–	–	2	–	1
G + *C* content range (%)	57	53.6	60.2	57	53.6	60.2	56	55.1	59.2

[a]*Pterocarya* is distributed in two regions of Iran: Hyrcanian and Zagrosian region.

Motif finding and secondary structure of ITS2

Simple sequence repeats in the ITS1 and ITS2 sequences were detected using the microsatellite repeat finder Web server (online at http://biophp.org/minitools/microsatellite_repeats_finder/demo.php).

A total of 49 (6 Iranian populations + 43 taxa from NCBI) sequences of ITS2 deposited in the NCBI GenBank were investigated (Supplemental Material). Homology-based RNA structure predictions of ITS2 related to Iranian taxa and *Pterocarya* samples of NCBI were conducted using the ITS2 database online software (http://its2.bioapps.biozentrum.uni-wuerzburg.de) (Wolf et al. 2008).

Biogeographic analysis and divergence time estimation

The distribution range of the *Pterocarya* genus was divided into three areas. These areas included A (Eastern Asia), B (Iran), and C (Turkey or Caucasus). We used the BEAST v1.8.2 software (Drummond & Rambaut 2007) to obtain phylogenetic trees (Maximum Parsimony) and the RASP software for biogeographic analysis (Yan et al. 2011). The Birth–Death Model and 10 million generations of the Markov chain Monte Carlo MCMC chains were run (1000 generations) in the BEAST v1.8.2 package. After 1000 trees, phylogeny were discarded as burn in, the samples were summarized by the maximum node heights supported above 0.95, using Tree Annotator v1.6.1 (Drummond & Rambaut 2007).

Results

ITS characteristics

The length and nucleotide composition of the ITS regions for Iranian *Pterocarya* are 644 bp without any polymorphisms (no nucleotide variations), but this region is variable from 644 to 651 for other species of the genus *Pterocarya*.

The ITS1 region of the data matrix had a very low pairwise divergence (1 and 10 sites for Iranian and all *Pterocarya*, respectively, Table I). These values fit within the known ranges for flowering plants, as reported by Baldwin et al. (1995).

Analysis of the DNA sequences from a broad range of taxa identified the angiosperm universal core motif in both 5.8S (GAATTGCAGAATCC) and ITS1 (GYGYCAAGGAA) in the genus *Pterocarya*.

Secondary structure and sequence repeats

The secondary structure characteristics of the ITS2 region of the *Pterocarya* taxa (both from Iranian forests and GeneBank) are shown in Table II. Helix III is the longest of all four, and Helix I presents the highest diversity among the different taxa (Table II).

The four domains show distinct size classes. Helix I, which is the most variable region of ITS2, is usually 9–21 bp long. Helix II varies from 7 to 18 bp, and Helix III ranges from 25 (*P. fraxinifolia*) to 42 bp (*P. fraxinifolia* and *P. macroptera*). Finally, Helix IV varies from 5 to 13 bp long.

The comparison of secondary structures based on nucleotide composition revealed that Helix 2 and Helix 4 are identical (100 nt), but are very different from Helix 1 and Helix 3 (Table III).

Repeats in the ITS1 and ITS2 sequences are shown in Table IV. Iranian *Pterocarya* has an exclusive motif GTGTGT both in the ITS1, position 113 and TS2, position143. These characteristics are peculiar of the Iranian samples *P. fraxinifolia* and are important for their differentiation from the other species of the genus *Pterocarya* located in East Asia, Iran, and Turkey (Table IV).

Phylogenetic relationships

The phylogenetic trees obtained in this study show that individuals of Pterocarya genus can be divided

Table II. Numerical and statistical values of the putative secondary structures (ITS2) determined in this study.

	Length (nt) in each helix				
Taxa	I	II	III	IV	Unpaired bases in total
IT1, IT2, LT7,LT10, PT1, HT3	20	36	44	26	76
P. hupehensis	18	36	48	26	77
P. macroptera var. delavayi	18	36	46	26	80
P. stenoptera	42	14	82	12	60
P. macroptera	42	14	84	12	60
P. fraxinifolia (from GenBank)	34	20	64	10	82
P. tonkinensis	34	20	66	10	90

Table III. Comparison of secondary structure of ITS2 of the species of the genus *Pterocarya* obtained from ITS2 database.

	Gl(model)	Species name	Helix 1	Helix 2	Helix 3	Helix 4
Pterocarya	10998591	*Pterocarya stenoptera*	90.476	100	100	100
	13568677	*Pterocarya tonkinensis*	100	100	100	100
	13568676	*Pterocarya fraxinifolia* (from GenBank)	100	100	100	100
	10998590	*Pterocarya macroptera*	90.476	100	95.238	100
Outgroup	10998593	*Cyclocarya paliurus*	85	100	97.561	100
Outgroup	223452035	*Juglans major*	89.474	100	95.122	100

Table IV. Sequence repeats found in the ITS1 and ITS2 sequences: software default (length of repeated sequence, 2–3; minimum number of repeats, 3; minimum length of tandem repeat, 6; percentage of mismatch, 0, 2).

Distribution		Positions							
		ITS1			ITS2				
		113	114	116	22	143	146	148	153
	Motif	gtgtgt	gtgtgt	gtgtgt	tgtgtg	gtgtgt	gtgtgt	gtgtgt	gtgtgt
	Sample								
Iranian samples	IT_1	+				+			
	IT_2	+				+			
	LT10	+				+			
	LT7	+				+			
	HT3	+				+			
	PT1	+				+			
East Asia	*P. hupehensis*		+		+			+	
	P. tonkinensis		+				+		
	P. stenoptera		+				+		
	P. macroptera			+					+
	P. macroptera var. delavayi		+				+		
Iran and Turkey	*P. fraxinifolia*			+					+

into three clades (Figure 2). Clade 1 includes the Iranian *P. fraxinifolia* populations and *P. fraxinifolia*, from the GenBank (AF179585) (alias *P. caucasica* in Figure 2). The species *P. hupehensis* and *P. macroptera* are grouped under clade 2, while *P. stenoptera* and *P. tonkinensis* are clustered in clade 3.

Pairwise genetic distance (Kimura's two parameter model, 1980) among all taxa ranged from 0.0 (between Iranian samples and *P. fraxinifolia* from the GenBank) to 0.0159 (between Iranian samples and *P. stenoptera* and *P. tonkinesis*) (Table V). Iranian populations show the minimum genetic distance with *P. fraxinifolia* from the GenBank and the maximum genetic distance (0.0175) with *P. stenoptera* and *P. tonkinensis* (Table V).

Network Analysis (Species relationships)

Figure 3 shows the relationships based on mutational steps among six taxa of the genus *Pterocarya* (5 species *P. fraxinifolia* from Iranian areas and gene Bank; *P. hupehensis*, *P. stenoptera*, *P. tonkinensis*, *P. macroptera*; and the variety *P. macroptera var. delavayi*). The statistical parsimony procedure revealed that *P. tonkinensis* and *P. stenoptera* are closely related. The Hyrcanian taxa and *P. fraxinifolia* from the GenBank (Table V) are closely related (2-bp difference only) to *P. hupehenis* and *P. macroptera*.

Biogeography analysis

The ancestral reconstruction at node 43 (ancestral node) suggests that ancestors of this group (All species from the genus *Pterocarya*) originated in area A (Figure 4), and this node is strongly supported with the posterior probability value of one. At node 40, dispersal events occurred from A to B (the most favored ancestral area is AB at this node). At node 35, a vicariance event caused the divergence of the *P. fraxinifolia* from the Caucasian region (its

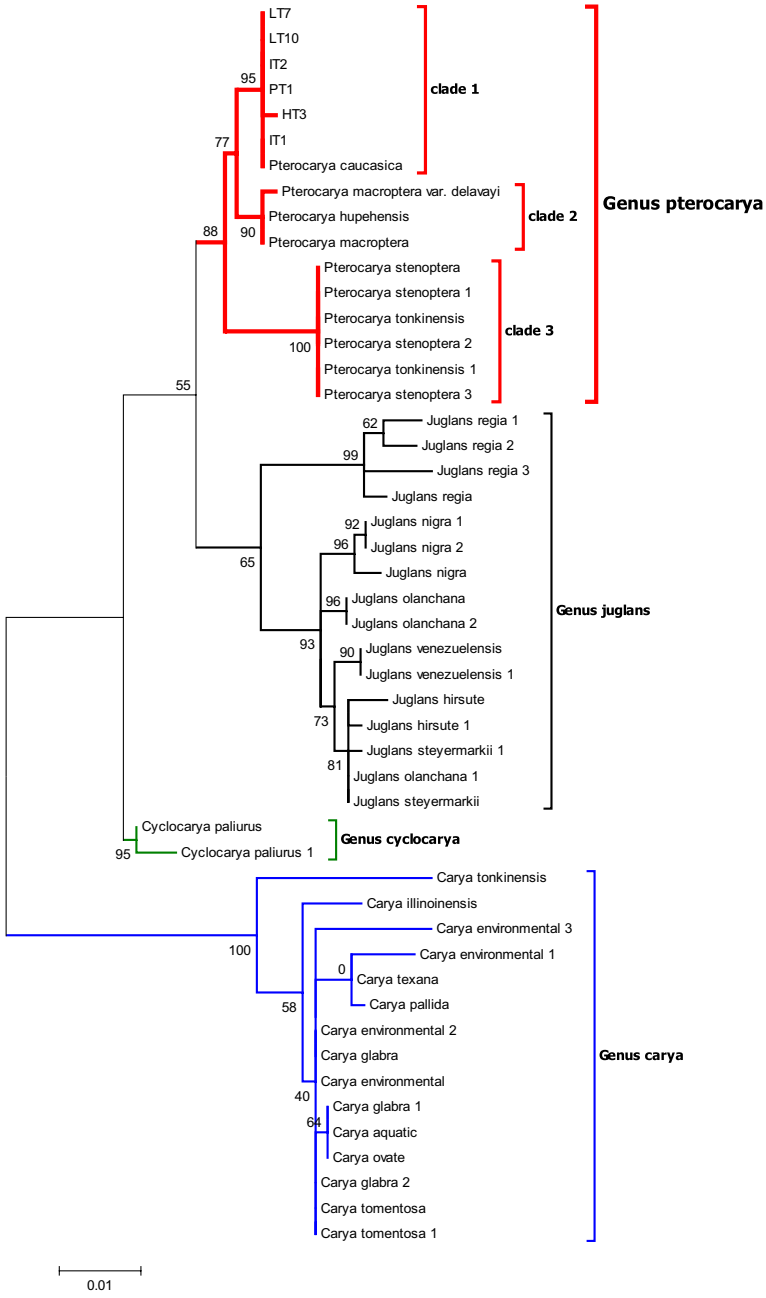

Figure 2. Maximum likelihood trees inferred from nrITS sequences of *Pterocarya fraxinifolia* populations detected in this study, with species of the genus *Pterocarya* from GenBank (asterisks) and *Cyclocarya*, *Carya* and *Juglans* as outgroups. Bootstrap values are reported on branches if higher than 50%.

Table V. Pairwise genetic distances between the *Pterocarya* taxa using Kimura-2-parameters distance based on ITS region.

	1	2	3	4	5	6	7	8	9	10	11
LT7	0.0000										
LT10	0.0000	0.0000									
IT2	0.0000	0.0000	0.0000								
PT1	0.0000	0.0000	0.0000	0.0000							
HT3	0.0016	0.0016	0.0016	0.0016	0.0000						
IT1	0.0000	0.0000	0.0000	0.0000	0.0016	0.0000					
P. hupehensis	0.0063	0.0063	0.0063	0.0063	0.0079	0.0063	0.0000				
P. macroptera var. delavayi	0.0079	0.0079	0.0079	0.0079	0.0095	0.0079	0.0016	0.0000			
P. stenoptera	0.0159	0.0159	0.0159	0.0159	0.0175	0.0159	0.0159	0.0175	0.0000		
P. tonkinensis	0.0159	0.0159	0.0159	0.0159	0.0175	0.0159	0.0159	0.0175	0.0000	0.0000	
P. macroptera	0.0063	0.0063	0.0063	0.0063	0.0079	0.0063	0.0000	0.0016	0.0159	0.0159	0.0000
P. fraxinifolia (from GenBank)	0.0000	0.0000	0.0000	0.0000	0.0016	0.0000	0.0063	0.0079	0.0159	0.0159	0.0063

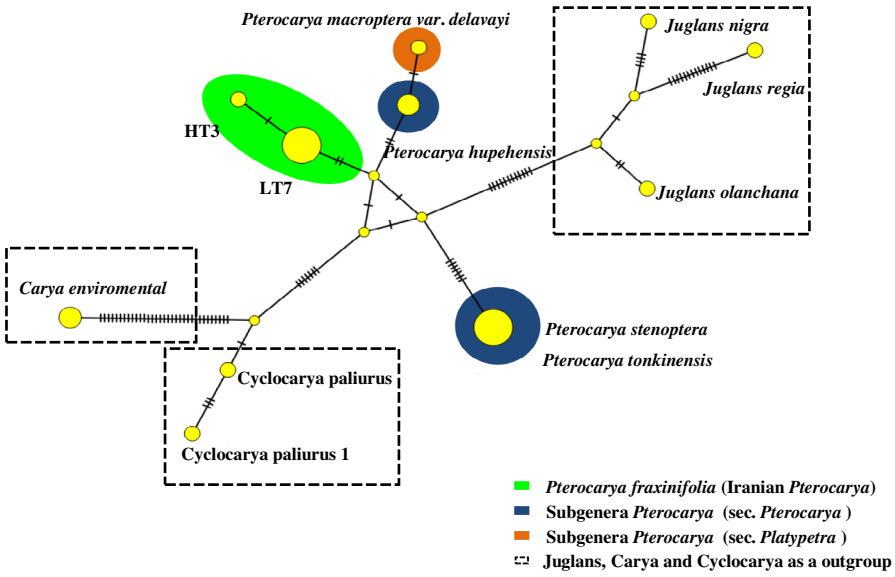

Figure 3. Phylogenetic network for nuclear nrITS sequences of the genus *Pterocarya*. Node sizes are proportional to the number of species belonging to the same SNP type (LT7 and HT3 labels are *Pterocaria fraxinifolia* samples from different regions of Iran).

sequence obtained from GenBank) and the Iranian populations.

Discussion

The genus *Pterocarya* is traditionally divided into two sections: *Pterocarya* and *Platyptera*. *Pterocarya* section contains four taxa: *P. fraxinifolia*, *P. hupehensis*, *P. stenoptera*, and *P. tonkinensis* (Rix 2007). This section is recognized by its buds (lack of bud scales) and having their male catkins lateral on old-growth twigs or scattered on new-growth twigs. There is a large disjunction between the East-Asiatic species and the single west Eurasian taxon, *P. fraxinifolia* (Rix 2007). The second section, *Platyptera*, includes the species of *P. rhoifolia* from Japan and *P. macroptera* with its three varieties (*var. macroptera*, *var. delavayi*, *var. insignis*) mainly distributed in China. Our analysis suggests, however, that this traditional division and phylogeny of the genus *Pterocarya* must be revised to account for all six species sampled throughout their distribution area (especially in Eastern Asia).

Surprisingly, the secondary structure of ITS2 and the sequence repeats were different among species of the genus *Pterocarya*. Although the *P. fraxinifolia* collected in Iran and obtained from the GenBank were in the same phylogenetic group (Figure 2, clade 1), they presented two different secondary structures and different sequence repeats (Table IV).

The reduction in the distribution area of *Pterocarya* in western Eurasia coincided with a phenomenon known as the "climate chaos of the Middle Ages", in which some areas of the earth experienced a significant increase in temperature and prolonged drought (Bradley et al. 2003).

Before the "climate chaos," the weather, probably very hot and humid with abundant precipitations, may have determined the wider distribution and frequency of *P. fraxinifolia* in Iran. Later, with the increasing temperatures and drier environments (a trans-regional phenomenon), only a few small and isolated stands of *P. fraxinifolia* survived outside the Hyrcanian forest area. Thus, except for two very small stands still present in western Iran, the main distribution of *P. fraxinifolia* became the Hyrcanian forest (north of Iran and southern Azerbaijan), with an annual rainfall of 1100 mm and 27 days of frost. The size of the two populations of *P. fraxinifolia* living in western Iran is very small, and with few individuals. The reductions in its distribution and population size may have promoted the genetic differentiation between these (Zagrossian area and Hyrcanian populations) disjunct populations.

In addition to geographical distance, the habitat conditions of the Zagrossian area and Hyrcanian forests, both including *P. fraxinifolia* populations, are very different. Indeed, the annual rainfall in western Iran is 684 mm with 90 days of frost, while in north of Iran and southern Azerbaijan annual rainfall is 1100 mm with 27 days of frost. The poor, rocky, and calcareous soil are the main differences between the isolated populations (Zagrossian populations) and Hyrcanian populations where the alluvial soils occur over a wide area of the

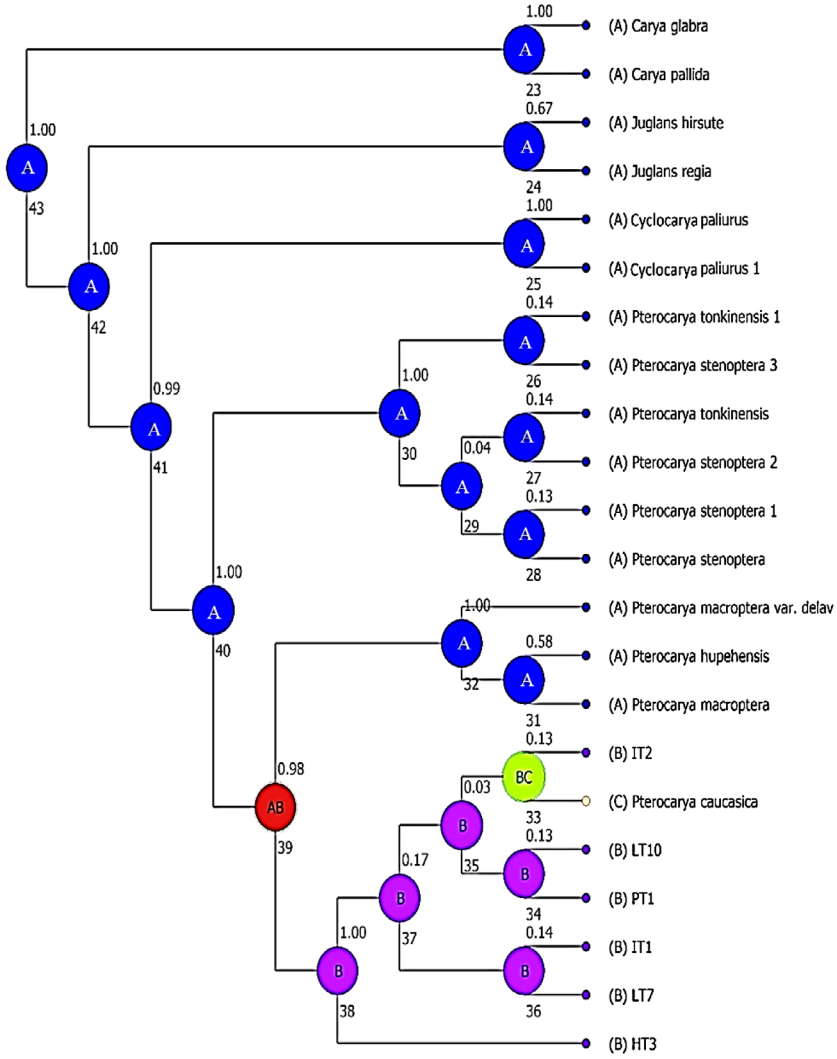

Figure 4. Ancestral state reconstructions ITS region of species *Pterocarya* with species of the genera *Cyclocarya*, *Carya* and *Juglans*. Based on statistical dispersal-vicariance analysis (S-DIVA) overlaid on the maximum clade credibility chronogram from BEAST. Current distributions are indicated in the center of each pie (A: East Asia; B: Iran; C: Turkey and Caucasus). Node ID and support values are shown at the bottom and top of each node, respectively. (IT2, LT10, PT1, IT1, HT3, LT7 labels are *Pterocaria fraxinifolia* samples from different regions of Iran).

plains and most river beds (Talebi et al. 2014). The adaptation of different populations to local environments could lead to genetic differentiation between populations. Indeed, Rix (2007) reported that in *P. macroptera*, the different geographical conditions likely led to the formation of three varieties. In contrast, our study does not support any genetic differentiation between the western Iran and Hyrcanian region using ITS and *trn*H-*psb*A markers. This could have occurred for two reasons: (1) insufficient time for the processes, such as genetic drift and inbreeding, that led to genetic erosion in small and isolated populations and cause a notable decrease in genetic diversity (Kaljund & Jaaska 2010). This was reported in fragmented populations of long-living trees (e.g. Young et al. 1993) and herbaceous perennials (e.g. Rosquist & Prentice 2000). (2) Alternatively, the DNA markers might not have sufficient discriminatory power. For example, according to its morphological characteristics and geographical distribution, *Alnus incana* was divided into four taxa, whereas ITS did not distinguish the subspecies from each other (Ren et al. 2010).

Biogeographic analysis showed that the Iranian and probably all other *P. fraxinifolia* populations in Western Eurasia, originated from East-Asiatic taxa via dispersal phenomenon. Based on this result, the Turkey and Caucasus populations most likely migrated through Iran by vicariance events.

Pollegioni et al. (2014) detected five obvious genetic barriers for Persian walnut and separated populations of western Asia (Georgia, Turkey, and Iran) from populations in Central and East Asia.

They expressed that the sharp genetic structure detected for Persian walnut across its Asian range attributed to repeated events of population fragmentation and genetic isolation after postglacial expansion and concluded that the complex topography of Central Asia and the transition climate from relatively humid to more arid conditions had major roles in shaping the observed phytogeographic regions of the Asiatic continent (Ricketts et al. 2001; Djamali et al. 2012). Additionally, analyzing the global distribution area of *P. fraxinifolia* drawn by Browicz (1982) (Supplemental data), *Pterocarya* might have arrived in Turkey and Caucasus via two routes: the first route could be from western Iran (Zagrossian forests) to the Mediterranean Sea, and the second from Iran (Hyrcanian forests) to Caucasus.

Conclusion

Based on the analysis of all six species considered throughout their distribution area, especially those in Eastern-Asia (Rix 2007), our results demonstrate that the traditional division and phylogeny of the genus *Pterocarya* should be revised. Additionally, the present study is the first attempt to elucidate the status and intraspecific relationships in *Pterocarya* genus from the Hyrcanian and Zagrossian forests of Iran. Although the *trn*H-*psb*A spacer has been discussed as a potential barcoding region (Kress et al. 2005; Vischi et al. 2006; Kress & Erickson 2007), it did not show any polymorphisms in the genus *Pterocarya*. However, we have found that the two main populations of *P. fraxinifolia* species (Iranians and from GeneBank) differed in their secondary structure and repeat sequences of ITS2 region, which was recently suggested to be a DNA barcode. The genetically isolated populations and clear gene barriers flow among the three main populations of *P. fraxinifolia* (Iran, Caucasus and Turkey) suggest the presence of independent population dynamics and permitting independent evolution of populations into different genotypes. Variation in the ITS regions have been observed between natural populations of some species. For example, Bobola et al. (1992) expressed that the differentiation of rDNA among populations of *Picea rubens* and *P. mariana* attributes to genetic drift or forces related to ecogeographical selection.

Further, molecular markers and morphological considerations are required to determine whether the Iranian *Pterocarya* (*P. fraxinifolia*) can be at least introduced as a distinct evolutionarily significant unit because it has different habitat ecological conditions and long-term separation from other *Pterocarya* populations.

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