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MOLECULAR DIVERSITY AND GENETIC RELATIONSHIPS BETWEEN *CARPINUS BETULUS* AND *C. ORIENTALIS* WITH INTER SIMPLE SEQUENCE REPEAT (ISSR) REGIONS

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A successful management and preservation of the natural populations depend on accurate assessment of genetic diversity. Knowing the genetic diversity within a population is important for choosing the conservation strategies for the species. The genus *Carpinus* belonging to Coryloideae, Betulaceae, has significant economic and ornamental importance. Determination of the taxa in the genus *Carpinus* in Iran is one of the most controversial issues among the researchers; for example, we can see this claim in the recent botanical literatures such as Sabeti and Browicz. However, two good species namely *C. betulus* L. and *C. orientalis* Mill. are the main species in Iran, adjacent regions and also in Europe. In general, taxonomic and biosystematics studies of the *Carpinus* are not known in Iran, moreover, in few cases, inter-specific hybrids and intermediate forms are recognised. A detailed molecular (ISSR) study of the *Carpinus* is done here with the following objectives: 1) to delimitate the species; 2) to carry out population genetic study and produce information on genetic structure, genetic variability within each population in *Carpinus betulus* and *C. orientalis*. In present study, 85 randomly collected plants from 17 geographical populations of two *Carpinus* species were considered. Our results indicated that ISSR markers can be used as a reliable and informative technique for evaluation of genetic diversity and relationships among *Carpinus* species.

Key words: *Carpinus*, ISSR, morphology, species delimitation

INTRODUCTION

The two most important components of biodiversity, species diversity and genetic diversity, have significant impact on ecosystem stability and resilience (Wehenkel *et al.* 2006). Forest succession is a fundamental ecological process in which the type of forest gradually changes over time and becomes stable (Byeon and Yun 2018). As the secondary succession species settle in relatively poor environments such as low nutrients and light in the stand compared to the early succession species, genetic diversity is an essential factor to

consider for the adaptability of the secondary species and the stability of the ecosystem after the succession (Wehenkel *et al.* 2011).

The birch family (Betulaceae) comprises six genera and approximately 167 species (Christenhusz and Byng 2016). In this family, the hornbeams in the genus *Carpinus* (Linnaeus 1753) are small to medium-size trees (Li and Skvortsov 1999, Holstein and Weigend 2017). About 35 species of the *Carpinus* genus have been reported worldwide: one species in North America, two species in Europe, and most species in Asia (Jeon *et al.* 2007). They are diploid species with 2n = 16 except *Carpinus betulus* (2n = 64) in Europe. Determination of the taxa in the genus *Carpinus* in Iran is one of the most controversial issues among the researchers; for example, we can see this claim in the recent botanical literature, such as Sabeti (1976) and Browicz (1972). However, two good species namely *C. betulus* L. and *C. orientalis* Mill. are the main species in Iran, adjacent regions (Mozaffarian 2005) and also in Europe. Intraspecific delimitation of *Carpinus orientalis* has been variously treated in different investigations. Some taxonomists believe the occurrence of two subspecies namely subsp. *orientalis* and subsp. *macrocarpa* Willk. (Browicz 1972), whereas others do not believe in any intraspecific classification within the species, but instead they consider them as two separate species (Sabeti 1976). *Carpinus orientalis*, a small and slow-growing deciduous tree/shrub, is different from its relative species, *C. betulus* due to having shorter and more base-branched trunks as well as growing mainly on rocky and poor sites (Browicz and Zielinski 1982, Sabeti 1976).

This species is distributed in SE Europe (Walterss 1964), Turkey, Caucasus and northern Iran, from west to easternmost of Hyrcanian forest especially in north of Khorassan province (Browicz 1972). It also disjunctly occurs in Semnan province (Mozaffarian 2005). According to the Flora Iranica (Browicz 1972), *C. orientalis* subsp. *orientalis* is distributed through northern Iran and also as a relatively small patch in Azerbaijan (Ali Bolaghi) and Khorasane Shomali (Bojnourd), while subsp. *macrocarpa* Willk. is distributed only in northern Iran (Gilan, Mazandaran and Golestan). The latter subspecies is an endemic taxon in the Iranian Hyrcanian forest zone and Talish in Azerbaijan Republic (Browicz 1972). *Carpinus betulus* L. (Betulaceae), is a common, late-successional, shade-tolerant tree often forming bushes and hedges. These edge communities between forest and pasture are highly valued for conservation due to their biodiversity. In addition, they provide refugia for plants and animals and connect biotopes. *Carpinus betulus* is also often used as an ornamental planting in gardens and non-forested landscapes. Sabety (2001), reported *C. betulus* var. *betulus* Browicz 1972, *C. betulus* var. *carpinizza* (Host) Neilr, *C. betulus* var. *parva* Radde-Fomin, and *C. betulus* var. *typica* Medo, all based upon leaf morphology in the Hyrcanian forest.

Genetic analyses in *C. betulus* are scarce; they were based on universal chloroplast markers (Grivet and Petit 2003) or anonymous amplified frag-

ment length polymorphisms (AFLPs; Coart *et al.* 2005). Microsatellite markers were established for several species within the family (Gürcan and Mehlenbacher 2010). Several phylogenetic studies were conducted to construct the infrageneric relationships, including those based on morphology (Hu 1964, Li and Cheng 1979, Winkler 1904), morphology and *rbc*L (Bousquet *et al.* 1992), *mat*K sequences (Kato *et al.* 1998), ITS sequences (Sun *et al.* 2010*a*, *b*) and ITS combined with morphology (Whitcher and Wen 2001, Yoo and Wen 2002). About the infrageneric relationship within the genus *Carpinus*, many botanists brought forward their own attitudes (Hu 1933, Winkler 1904). Genetic diversity and differentiation in two *Carpinus* species (*C. betulus and C. orientalis*) occurring in Romania was investigated by using three chloroplast Simple Sequence Repeat markers (cpSSRs) (Cărăbuş *et al.* 2017). RST values for both species suggest low levels of recurrent gene flow through seeds among populations. Molecular markers provide a powerful tool for studying the genetic diversity. Among advanced genetic markers, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers have been widely used for diversity analyses (Pharmawati *et al.* 2004). RAPD technique is quick, easy and requires no prior sequence information. The technique detects nucleotide sequence polymorphism using a single primer of arbitrary nucleotide sequence (Moreno *et al.* 1998). ISSR marker involves PCR amplification of DNA by a single 16–18 bp. long primer composed of a repeated sequence anchored at the 3′ or 5′ end of 2–4 arbitrary nucleotides. The technique is rapid, simple, inexpensive and more reproducible than RAPD (Esfandani-Bozchaloyi *et al.*, 2018*a*, *b*, *c*, Esfandani-Bozchaloyi and Sheidai 2018, Godwin *et al.* 1997, Zietkiewicz *et al.* 1994).

The present research was undertaken with the aims of evaluating the genetic diversity of the 2 species of *Carpinus*. We try to answer the following questions: 1) Is there infra and interspecific genetic diversity among studied species? 2) Is genetic distance among these species correlated with their geographical distance? 3) What is the genetic structure of populations and taxa? 4) Is there any gene exchange between *Carpinus* species in Iran?

MATERIALS AND METHODS

Plant materials. A total of 85 individuals were sampled representing 17 natural populations in East Azerbaijan, Mazandaran, Golestan, Gilan Provinces of Iran (Table 1). In the present study, 40 plant samples were collected from 8 geographical populations of *Carpinus orientalis* and 45 plant samples were collected from 9 geographical populations of *Carpinus betulus*. Different references were used for the correct identification of species (*Carpinus betulus* and *Carpinus orientalis*), (Browicz 1972, Sabeti 1976). Details of sampling sites are mentioned in Table 1 and Figure 1. On the other hand, we studied her-

Table 1
Location and herbarium accession numbers of the studied populations (P.) of *Carpinus betulus* and *Carpinus orientalis* collected by Riyahee in Iran

P.	Locality	Latitude, longitude	Alt. (m)	Voucher no.
		C. betulus		
1	Mazandaran, Chamestan toward waz forest	36.0111° N, 52.1326°E	174	IAUH-000014976
2	Mazandaran, Kheyroud-Kenar forest	36.5412°N, 51.5927°E	118	IAUH-000014977
3	Mazandaran, Nowshahr, Madan forest	36.42393°N, 51.2392°E	113	IAUH-000014978
4	Mazandaran, Siahbisheh	36.62393°N, 51.2592°E	1,759	IAUH-000014979
5	Gilan, Hashtpar	39.52393°N, 50.3592°E	1,730	IAUH-000014980
6	Golestan, Golestan National Park	37.42393°N, 55.7592°E	785	IAUH-000014981
7	Golestan, Gorgan Ziarat road	36.5151°N, 54.0228°E	931	IAUH-000014982
8	Golestan, Gorgan, Zarrin Gol	36.52393°N, 54.2392°E	215	IAUH-000014983
9	E Azerbaijan, Kaleybar to Ghaleh Babak	38.0111°N, 47.2326°E	1,096	IAUH-000014984
		C. orientalis		
1	Mazandaran, Siah Bisheh	36.12393°N, 51.3592°E	2,017	IAUH-000014985
2	Mazandaran, Chalus, Hezar Cham	36.72313°N, 51.1592°E	1,376	IAUH-000014986
3	Mazandaran, Chalus, between Dozdbon and Delir	36.52395°N, 51.2542°E	1,150	IAUH-000014987
4	Mazandaran, Kojur, Dasht Nazir	36.22393°N, 51.6552°E	963	IAUH-000014988
5	Mazandaran, Kojour, Otaghsara village	36.62393°N, 51.7512°E	1,647	IAUH-000014989
6	Golestan, Gorgan, Radkan road	36.92391°N, 59.1522°E	1,205	IAUH-000014990
7	Golestan, 22 km Azad Shahr to Shahroud	36.12391°N, 55.3532°E	479	IAUH-000014991
8	Golestan, 30 km Azad Shahr to Shahroud	36.52392°N, 55.2592°E	601	IAUH-0000149922

barium specimens from TARI and HNBG (Herbarium of Nowshahr Botanical Garden) that were considered as seventeen populations (Table 1).

DNA extraction and ISSR assay. Fresh leaves were used randomly from 5–10 plants in each of the studied populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA (Esfandani-Bozchaloyi *et al.* 2019). The quality of extracted DNA was examined by running on 0.8% agarose gel. For the ISSR analysis, 22 primers from UBC (University of British Columbia) series were tested for DNA amplification. Ten primers were chosen for ISSR analysis of genetic variability, based on band reproducibly (Table 2). PCR reactions were carried in a 25 μl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 μM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The amplifications' reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94 °C, followed by

Table 2
Details about the banding pattern revealed by ISSR primers

Primer name	Primer sequence (5′–3′)	TNB	NPB	PPB (%)	PIC	PI	EMR	MI
ISSR-1	DBDACACACACACACACA	10	10	100.00	0.38	5.86	7.55	2.45
ISSR-2	GGATGGATGGATGGAT	11	10	91.00	0.48	4.91	7.43	2.85
ISSR-3	GACAGACAGACAGACA	12	10	83.00	0.36	5.34	8.55	3.44
ISSR-4	AGAGAGAGAGAGAGAGYT	10	10	100.00	0.43	4.88	8.56	3.65
ISSR-5	ACACACACACACACACC	13	13	100.00	0.25	5.23	7.23	2.47
ISSR-6	GAGAGAGAGAGAGAGARC	11	9	91.00	0.35	4.66	7.56	2.67
ISSR-7	CTCTCTCTCTCTCTCTG	13	10	77.00	0.44	3.21	9.60	4.55
ISSR-8	CACACACACACACACAG	13	11	92.00	0.32	4.32	9.55	4.45
ISSR-9	GTGTGTGTGTGTGTGTYG	12	10	83.00	0.45	4.56	7.34	2.11
ISSR-10	CACACACACACACACARG	11	10	91.00	0.47	4.25	7.11	2.87
	Average	11.6	10.5	90.00	0.39	4.72	7.66	3.1

Note: TNB = the number of total bands; NPB = the number of polymorphic bands; PPB (%) = the percentage of polymorphic bands; PI = polymorphism index; EMR = effective multiplex ratio; MI = marker index; PIC = polymorphism information content for each of CBDP primers

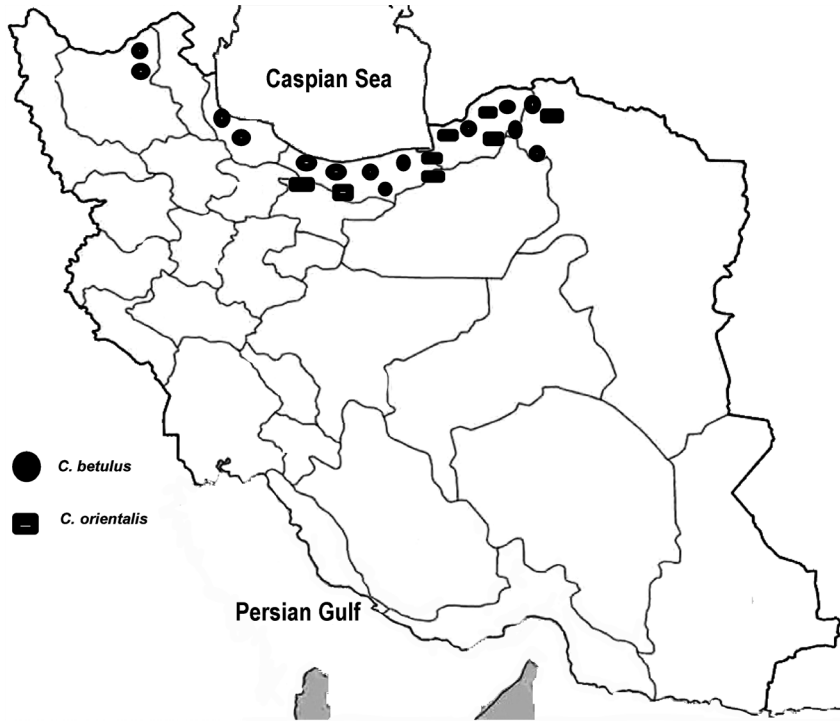

Fig. 1. Distribution map of the populations studied

40 cycles of 1 min at 94 °C; 1 min at 52–57 °C and 2 min at 72 °C. The reaction was completed by final extension step of 7–10 min at 72 °C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses. Molecular analyses – ISSR bands obtained were coded as binary characters (presence = 1, absence = 0) and used for genetic diversity analysis. Genetic diversity parameter of Nei's gene diversity (H), Shannon information index (I), number of effective loci, and percentage of polymorphism (Freeland *et al.* 2011, Weising *et al.* 2005), were determined by GenAlEx 6.4 (Peakall and Smouse 2006). Nei's genetic distance among populations was used for Neighbour Joining (NJ) clustering and Neighbour-Net networking (Freeland *et al.* 2011, Huson and Bryant 2006). Mantel test checked the correlation between geographical and genetic distance of the studied populations (Podani 2000). These analyses were done by PAST ver. 2.17 (Hammer *et al.* 2012), Darwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) software. AMOVA (Analysis of molecular variance) test (with 1,000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse 2006), and Nei's Gst analysis as implemented in GenoDive ver.2 (2013) (Meirmans and Van Tienderen 2004) were used to show genetic difference of the populations. Moreover, populations' genetic differentiation was studied by G'ST est = standardised measure of genetic differentiation (Hedrick 2005), and D_est = Jost measure of differentiation (Jost 2008).

The genetic structure of populations was studied by Bayesian based model STRUCTURE analysis (Pritchard *et al.* 2000), and maximum likelihood-based method of K-Means clustering of GenoDive ver. 2. (2013). For STRUCTURE analysis, data were scored as dominant markers (Falush *et al.* 2007). The Evanno test was performed on STRUCTURE result to determine proper number of *K* by using delta *K* value (Evanno *et al.* 2005). In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), provide the best fit for k (Meirmans 2012). Gene flow was determined by (i) Calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm = 0.5(1–Gst)/Gst.

RESULTS

Species delimitation in Carpinus betulus *and* C. orientalis

Ten ISSR primers were screened to study genetic relationships among *Carpinus* species; all the primers produced reproducible polymorphic bands in *Carpinus betulus* and *Carpinus orientalis.* A total of 105 amplified polymorphic bands were generated across 2 *Carpinus* species. The size of the amplified fragments ranged from 100 to 2,500 bp (Table 2).

Table 3
Genetic diversity parameters in the studied populations of *C. betulus* (N = number of samples, Ne = number of effective alleles, I = Shannon's information index, He = gene diversity, uHe = unbiased gene diversity, P% = percentage of polymorphism, populations). Populations 1–9 are according to Table 1

	N	Na	Ne	I	He	uHe	P%
Pop1	5	0.524	1.127	0.111	0.073	0.082	21.68
Pop2	5	0.469	1.108	0.102	0.066	0.073	21.68
Pop3	5	0.406	1.122	0.100	0.068	0.076	17.48
Pop4	5	0.343	1.080	0.068	0.046	0.051	12.59
Pop5	5	0.322	1.061	0.063	0.039	0.044	14.69
Pop6	5	0.483	1.104	0.102	0.065	0.072	22.38
Pop7	5	0.315	1.061	0.061	0.039	0.043	13.29
Pop8	5	0.252	1.040	0.034	0.023	0.025	6.29
Pop9	5	0.364	1.063	0.063	0.040	0.044	13.99

Different clustering and ordination methods produced similar results. NJ clustering and MDS plot (Figs 2–3), of the studied populations did not entirely delimit the studied species and revealed that plants in these species are intermixed. In NJ dendrogram, a higher degree of intermixture occurred between *Carpinus betulus* and *C. orientalis.*

MDS plot (Fig. 3) showed that a high degree of intraspecific genetic variability as they are positioned in different places of the plot.

Intra-specific variation of Carpinus betulus

Genetic diversity parameters determined in 9 geographical populations of *Carpinus betulus* are presented in Table 3. The highest value of polymor-

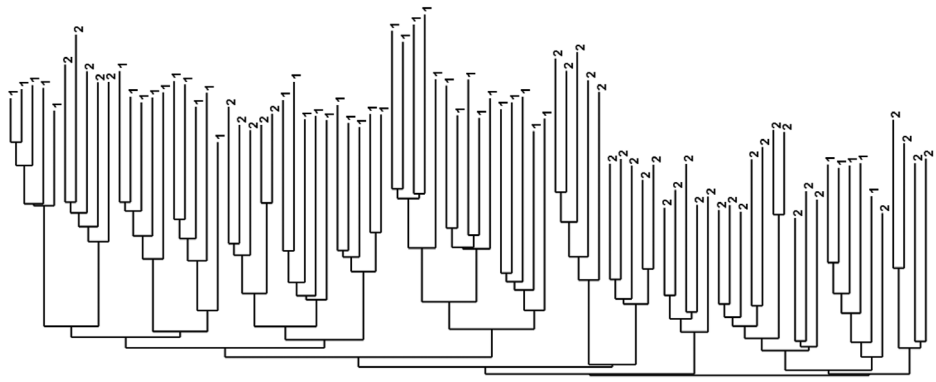

Fig. 2. NJ tree based on ISSR markers. Abbreviations: 1–2 are *C. betulus* (1); *C. orientalis* (2)

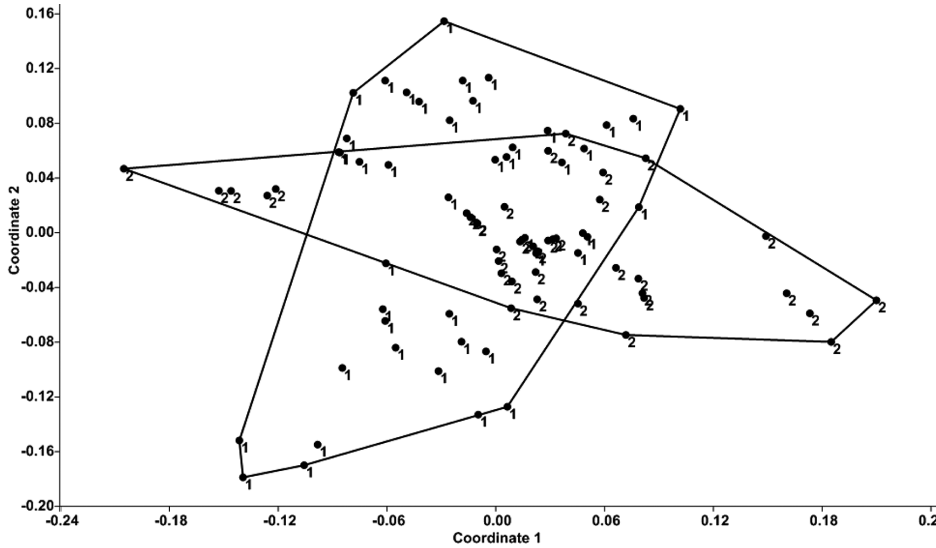

Fig. 3. Multidimensional scaling plot based on based on ISSR markers. Abbreviations: 1–2 are *C. betulus* (1); *C. orientalis* (2)

phism percentage (22.38%) occurred in population Golestan, Golestan National Park that also had a high value for gene diversity (0.065) and Shannon' information index (0.102). Population Golestan, Gorgan, Zaringol has the lowest value for the percentage of polymorphism (6.29%) and the lowest value for Shannon' information index (0.034), and gene diversity (He = 0.023).

Significant molecular difference (P = 0.01) was obtained among the studied population by AMOVA that was supported by Gst analysis (0.362, P =

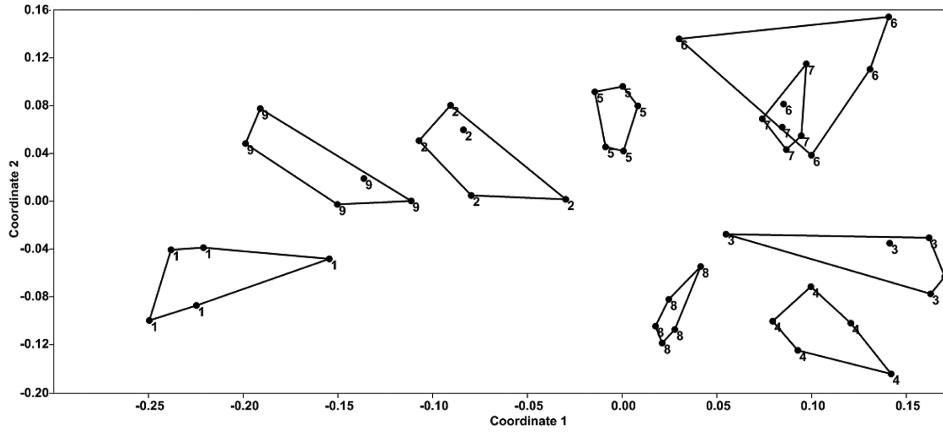

Fig. 4. MDS plot of ISSR data in *C. betulus* populations studied

Table 4
AMOVA test of the studied populations *Carpinus betulus*

Source	df	SS	MS	Est. Var.	%
Among populations	8	366.178	45.772	8.110	61
Within populations	36	188.000	5.222	5.222	39
Total	44	554.178		13.332	100
Stat	Value	P(rand ≥ data)			
PhiPT	0.608	0.001			

0.001). Moreover, significant values for Hedrick standardised fixation index after 999 permutation (G'st = 0.142, P = 0.001) and Jost' differentiation index (D-est = 0.175, P = 0.001), indicate that the geographical populations of *Carpinus betulus* are genetically differentiated. AMOVA revealed that 61% of total genetic variability occurred among the studied populations while 39% occurred within these species Table 4.

MDS plots of ISSR data (Fig. 4) separated the studied populations from each other. The plot also showed the presence of a higher within population genetic diversity in the populations Golestan, Golestan National Park.

Mantel test after 5,000 permutations produced significant correlation between genetic and geographical distance in these populations (r = 0.35, P = 0.0002). Therefore, the populations that are geographically more distant have less amount of gene flow, and we have isolation by distance (IBD) in *C. betulus*.

The comparison between genetic identity and genetic distance (table not given) showed a genetic similarity (0.94) between populations Mazandaran, Siahbisheh and Gilan, Hashtpar, while the lowest genetic similarity value (0.84) occurs between Mazandaran, Chamestan to Ward Waz forest and Golestan, Gorgan, Zarrin Gol populations.

K-Means clustering result showed that the best clustering (optimum number of genetic groups = k) according to Calinski and Harabasz' pseudo-F was k = 2 (the highest value of pseudo-F = 8.554). The optimum number of k according to Bayesian Information Criterion was 2 (the lowest value of BIC = 867.9). Similar result was obtained by Evanno test performed on STRUCTURE analysis which produced a major peak at k = 2. STRUCTURE plot based on k = 2 (Fig. 5), revealed that populations 1, 2, and 9 are distinct in their genetic

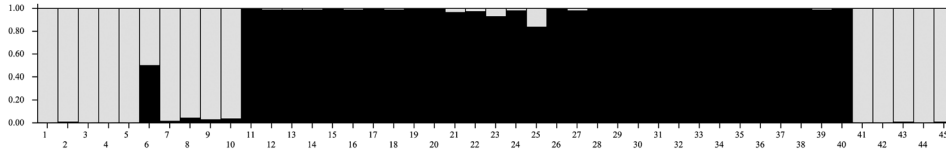

Fig. 5. STRUCTURE plot of *C. betulus* populations based on k = 2 of ISSR data

Table 5
Genetic diversity parameters in the studied populations of *C. orientalis* (N = number of samples, Ne = number of effective alleles, I = Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P% = percentage of polymorphism, populations). Populations 1–8 are according to Table 1

	N	Na	Ne	I	He	uHe	P%
Pop1	5	0.434	1.095	0.084	0.056	0.062	16.08
Pop2	5	0.357	1.068	0.069	0.044	0.049	14.69
Pop3	5	0.490	1.123	0.109	0.072	0.080	20.98
Pop4	5	0.259	1.051	0.053	0.033	0.037	11.89
Pop5	5	0.455	1.115	0.109	0.071	0.079	22.38
Pop6	5	0.273	1.052	0.051	0.033	0.036	11.19
Pop7	5	0.329	1.086	0.081	0.053	0.059	16.08
Pop8	5	0.238	1.057	0.053	0.035	0.038	10.49

content (were differently coloured), while populations 3 to 8, had some degree of shared alleles. This genetic grouping is not in agreement with MDS plot result presented before. The reticulograms obtained revealed gene flow/shared alleles among most of the studied populations (figure not given). The mean Nm = 0.22 was obtained for all ISSR loci, which indicates low amount of gene flow among the populations and supports genetic stratification as indicated by MDS analyses.

Intra-specific variation of Carpinus orientalis

Genetic diversity parameters determined in 8 geographical populations of *Carpinus orientalis* are presented in Table 5. The highest value of percentage polymorphism (22.38%) was observed in Mazandaran, Kojur Otaghsara village, which shows high value for gene diversity (0.071) and Shannon' information index (0.109). Population Golestan, 30 km Azad Shahr to Shahroud has the lowest value for the percentage of polymorphism (10.49%) and the lowest value for Shannon, information index (0.053), and He (0.035).

The significant molecular difference (P = 0.01) are highlighted by the AMOVA test. 54% and 46% results, respectively, as inter- and intra-population genetic variability. These results indicate that the geographical populations of *Carpinus orientalis* are genetically differentiated from each other (Table 6).

PCA plot of ISSR data (Fig. 6) showed both intra- and inter-population genetic diversity and revealed close genetic affinity between populations 2, 5. The plots showed higher within population genetic diversity in the populations Mazandaran, Siahbisheh. The studied population were placed in a sepa-

Table 6
AMOVA test of the studied populations *Carpinus orientalis*

Source	df	SS	MS	Est. Var.	%
Among populations	8	288.711	36.089	6.160	54
Within populations	36	190.400	5.289	5.289	46
Total	44	479.111		11.449	100
Stat	Value	P(rand ≥ data)			
PhiPT	0.538	0.001			

rate group which was in agreement with AMOVA result. Mantel test after 5,000 permutations produced significant correlation between genetic distance and geographical distance in these populations (r = 0.45, P = 0.0002). Therefore, the populations that are geographically more distant have less amount of gene flow, and we have isolation by distance (IBD) in *Carpinus orientalis*.

The comparison between genetic identity and genetic distance (figure not given), showed a genetic similarity (0.97) between populations Mazandaran, Kojur, Dasht Nazir and Golestan, 30 km Azad Shahr to Shahroad, while the lowest genetic similarity value (0.85) occurs between Mazandaran, Siahbisheh and Mazandaran, Chalus between Dozdbon and Delir.

The NJ tree (Fig. 6), produced two major clusters. Mazandaran, Kojur, Dasht Nazir and Golestan, 22 km Azad Shahr to Shahroud and Golestan, 30 km Azad Shahr to Shahroud showed close genetic affinity and formed the first cluster. While other populations were placed in a single cluster far from the other studied populations. NJ tree supported the grouping made by PCA plot (Fig. 7).

STRUCTURE plot based on k = 2, was performed with admixture model ancestry and revealed genetic affinity between Mazandaran, Siahbisheh and Mazandaran, Chalus, Hezar Cham as well as 2 and 4–8 due to shared common alleles (Fig. 8). This is not in agreement with NJ tree and PCA plot presented

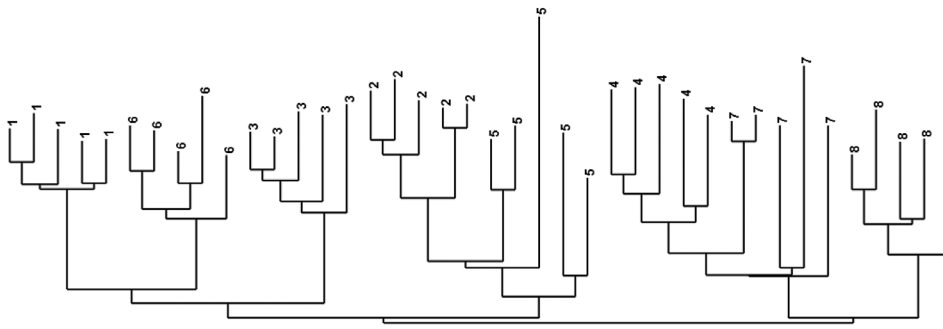

Fig. 6. NJ tree of ISSR in *C. orientalis* populations studied. Different colours indicate the plant specimens (Nos 1–8) studied from each geographical population

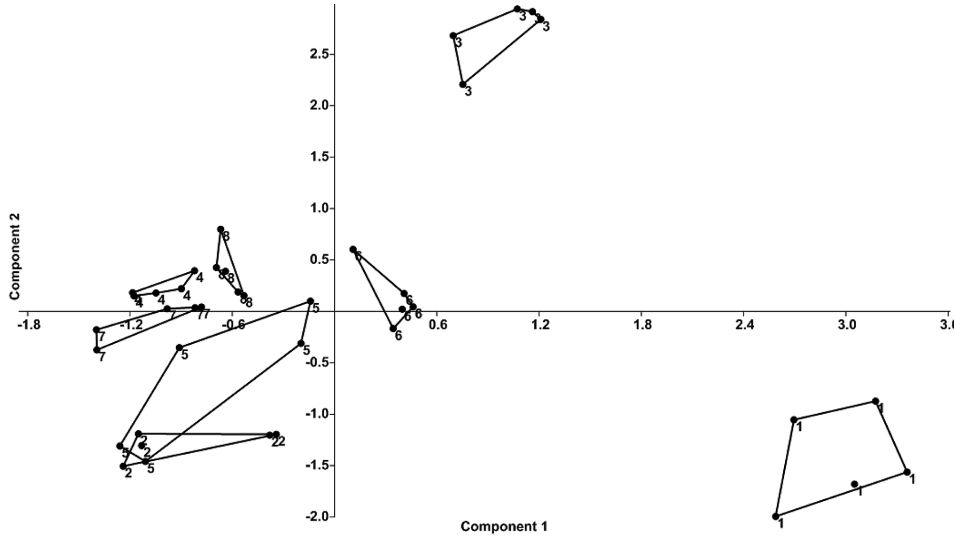

Fig. 7. PCA plot of ISSR in *C. orientalis* populations studied. Different colours indicate the plant specimens (Nos 1–8) studied from each geographical population

before. Both these analyses revealed that *Carpinus orientalis* populations show genetic stratification.

The reticulogram obtained (Nm = 0.32; figure not given), supported low degree of gene flow/ancestral shared sequences between 4 and 5, as well as between 4 and 1, 2. This result is in agreement with STRUCTURE plot of the studied species based on ISSR nuclear data and all these results are in agreement in showing high degree of genetic stratification within *Carpinus orientalis* populations.

DISCUSSION

Species delimitation and taxonomic consideration

Plant species delimitation and infra-specific genetic diversity are two important areas of investigation in phylogenetic systematics, evolution, biogeography and biodiversity studies. Data obtained can help to understand the patterns and mechanisms of speciation and hybridisation (Esfandani-Bozch-

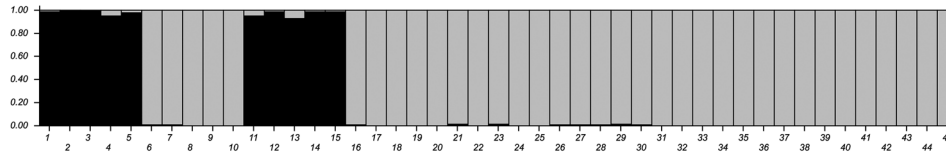

Fig. 8. STRUCTURE plot of *C. orientalis* populations based on k = 2 of ISSR data

aloyi *et al.* 2017*a*, *b*, *c*, *d*). They can reveal the pattern of gene flow between closely related phylogenetic species versus isolation by distance and identify the evolutionary process by which new biological species arise (Freeland *et al.* 2011). Species delimitation is a difficult task particularly in the species with cross-pollination breeding system that tend to form frequent inter-specific hybrids (Esfandani-Bozchaloyi *et al.* 2017*b*, 2018*a*, *b*).

For this purpose we collected plants of *Carpinus betulus* and *Carpinus orientalis* from the areas they grow and the areas of overlap and delimit these two species. Studying intra-specific variability in these two species is another aims of present study therefore we undertook population genetic investigation of the mentioned species. In Iran, the Alborz Mts, between the Caspian Sea and the Iranian Plateau, create an environment extremely propitious to the growth of a unique flora in the Hyrcanian Forest. The Hyrcanian Forest is part of the Euro-Siberian region, and is rich in broadleaf species (about 80 tree and 50 shrub species), while only three to four conifer species. Ecological conditions such as soil, temperature, rainfall and solar radiation vary considerably and visibly with increasing altitudes (Körner 2007, Wang *et al.* 2003). Hornbeam (*Carpinus betulus*) is one of the most valuable tree species in the Hyrcanian forest and occurs across many elevations from the coastal plain at sea level to an altitude of around 1,800 m in *Quercus-Carpinetum* and *Parrotia-Carpinetum* communities (Sagheb-Talebi *et al.* 2003). Our study attempted to estimate genetic diversity, genetic differentiation and genetic structure of *Carpinus betulus* and *Carpinus orientalis* in Iran by using ISSR analysis. The level of genetic diversity was high compared with same genus species and similar life history traits. The value of genetic variation of *C. betulus* and *C. orientalis* have been investigated with different DNA markers targeting different polymorphic regions of the chloroplast genome by PCR-RFLP (Restriction Fragment Length Polymorphism) (Postolache *et al.* 2017) and also by using chloroplast microsatellites (Simple Sequence Repeats; cpSSRs) (Fărcaş *et al.* 2006, Grivet and Petit 2003). The chloroplast DNA diversity for *C. betulus* was significantly lower in western Europe compared with SE European populations, that harbour nearly all genetic variation and consequently a more detailed analyses in this region is absolutely necessary to quantify the genetic structure and diversity. Beside this, previously published results Grivet and Petit (2003) indicate for very low introgression between these two species. The *C. betulus* populations from Romania have a very distinct Holocene postglacial history with direct consequences on current genetic pattern that needs to be investigated further in order to understand the species diversity and evolutionary history (Fărcaş *et al.* 2006, Grivet and Petit 2003). Moreover, the phylogeographical data on *C. orientalis* are very scarce and these data are very important in the conservation and management of this drought-tolerant woody species. Ahn *et al.* (2019) applied eight primer-restriction enzyme combinations to investigate genetic diversity, genetic differentiation, and genetic structure of *Carpinus laxi-*

flora populations with AFLP markers. The level of genetic differentiation was very small compared to that of *Carpinus* species and other species with a similar life history. The degree of genetic variability within a species is highly correlated with its reproductive mode, the higher degree of open pollination/ cross breeding brings about higher level of genetic variability in the studied taxon (Freeland *et al.* 2011). There were no reports about mating system studies of *Carpinus* species. Most flowering plants are hermaphroditic which are known to have mixed mating (Whitehead *et al.* 2018). The ratio between outcrossing and selfing vary in accordance with species or environment of the stand. Plants have evolved to increase outcrossing in many ways such as self-incompatibility, sexual strategy, and pollen capture (Friedman and Barrett 2009). It is known that wind pollination is an evolutionary strategy of animal or insect pollination. The results from mating system studies of 267 species revealed that wind-pollinated species was higher than those of animal- or insect-pollinated species (Goodwillie *et al.* 2005).

NJ clustering and MDS plot of the studied populations based on ISSR markers did not entirely delimit the studied species and revealed that plants in these species are inter-mixed. In NJ dendrogram, a higher degree of intermixture occurred between *C. betulus* and *C. orientalis.*

The present study also revealed significant genetic difference among *C. betulus* and *C. orientalis* populations, quite in agreement with the mentioned assumption. This is particularly supported by STRUCTURE plot that identified separate genetic groups within this population. Different mechanisms like isolation, drift, founder effects and local selection may act to bring about among population differentiation and therefore, populations differ in phenotypic traits and allelic composition (Jolivet and Bernasconi 2007).

Some taxonomists believe the occurrence of two subspecies namely subsp. *orientalis* and subsp. *macrocarpa* Willk. (Browicz 1972), whereas others do not believe in any intraspecific classification within the species, but instead they consider them as two separate species (Sabeti 1976). According to the Flora Iranica (Browicz 1972), *C. orientalis* subsp. *orientalis* is distributed through northern Iran and also as a relatively small patch in Azerbaijan while subsp. *macrocarpa* Willk. is distributed only in northern Iran (Gilan, Mazandaran and Golestan). Sabety (2001) reported *Carpinus betulus* var. *betulus*, *C. betulus* var. *carpinizza* (Host) Neilr, *C. betulus* var. *parva* Radde-Fomin, and *C. betulus* var. *typica* Medo, all based upon leaf morphology in the Hyrcanian forest. Also according to the Flora Iranica (Browicz 1972) reported *Carpinus betulus* var. *betulus* and *Carpinus betulus* var. *parva,* our results based on STRUCTURE plot showed k = 2 based on genetic data separated some of these populations from the others suggesting the existence of ecotypes or subspecies within these species.

The present population divergence may be under influence of isolation-by distance across the distribution range of the studied populations. The dispersal of these populations might be constrained by distance and gene flow

is most likely to occur between neighbouring populations. As a result, more closely situated populations tend to be more genetically similar to one another (Medrano and Herrera 2008). The populations' divergence may be accompanied by local adaptation. When we use multilocus molecular markers (such as SSR, AFLP, RAPD, ISSR, etc.) for population genetic studies we understand that these are neutral molecular markers (they are not directly acting as adaptive genes), but they may be linked to a gene or a genetic region with adaptive value (Freeland *et al.* 2011).

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