J. For. Res. (2019) 30(2):451–461
https://doi.org/10.1007/s11676-018-0785-4

ORIGINAL PAPER

Phylogenetic relationship and genetic differentiation of *Populus caspica* and *Populus alba* using cpDNA and ITS noncoding sequences

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Received: 8 October 2016 / Accepted: 18 September 2017 / Published online: 18 September 2018

Abstract *Populus caspica* Bornm. (section *Leuce* and subsection *Albida*), one of the most endangered endemic tree species in the Hyrcanian Forest in Iran, has numerous morphological characteristics that are closely similar to *Populus alba*; to clarify their taxonomic relatedness and genetic differentiation and thus inform conservation strategies, we used the noncoding regions of chloroplast DNA (cpDNA; *trnL-F* and *trnH-psbA*) and the internal transcribed spacer (ITS). Leaf samples were collected from six populations across northern Iran. cpDNA and ITS fragments were amplified by universal primers using the PCR technique and directed sequencing. The results showed that *P. caspica* is genetically differentiated from *P. alba*, and two ITS variants were detected within some *P. caspica* individuals. Conflicts between topologies from ITS and plastid genomes were observed. High differentiation of *P. caspica* from the other *Populus* species shown in this study confirmed the diverging taxonomic status of this endangered species. We recommend in situ conservation measures (e.g., protected areas) for at least several populations of this species, especially in the plain regions of the Hyrcanian forest.

Keywords Endangered endemic species · DNA barcoding · Hyrcanian forest · Taxonomic status

Introduction

Very important forest trees such as aspens, cottonwoods, and poplars, belong in the genus *Populus* (Salicaceae), commonly called poplars, they are mainly distributed in northern subtropical forests and play a significant ecological role as an indicator species in these regions (Dickmann et al. 2001; Hamzeh and Dayanandan 2004). They are long-lived trees characterized by a dioecious breeding system and wind dispersal of pollen and seeds, and are also commonly used as the model species for tree genomics (Tuskan et al. 2006).

The Hyrcanian forest in northern Iran is located between the Caspian Sea and the Alborz Mountains. These forests comprise 15% of the Iranian forests and represent 1.1% of the country's land area (Yousefzadeh et al. 2012). This region has a unique rich biodiversity and is home to many endemic species. Although recognized as a refuge for many Arco-Tertiary relict plants, many forest habitats, and especially the riverine vegetation in the Caspian forest, have been greatly degraded by human activities (Leestmans 2005; Akhani et al. 2010).

Project funding: This project is supported by grant funding from the Tarbiat Modares University and Mazandaran University.

The online version is available at http://www.springerlink.com

Corresponding editor: Hu Yanbo.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11676-018-0785-4) contains supplementary material, which is available to authorized users.

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Populus caspica Bornm., one of the most-endangered relict tree species of the Hyrcanian forest (Fallah et al. 2012), is a fast-growing hygrophytic tree, distributed throughout the Hyrcanian forest from the plains to 1400 m above sea level. Unfortunately, many of these habitats have been destroyed because of land-use changes, illegal exploitation and habitat fragmentation (Colagar et al. 2013). Large-scale interspecific hybridization and high levels of morphological variation among poplars have resulted in great difficulty in the classification of species in systematic and comparative evolutionary research.

Populus caspica has very similar morphological features with *P. alba* and is differentiated mainly by the inflorescence and petiole sizes; the petiole of *P. caspica* is 10–15 cm long (*P. alba* 10 cm) and inflorescence is 20 cm long (*P. alba* 10–15 cm). *Populus alba* has many ridges in its bark and grows in the sun, tolerates almost any soils, wet or dry, and is distributed in the river areas of southern Europe, north Africa and central Asia; this species can be found in active floodplains (Hamzeh and Dayanandan 2004). *Populus caspica*, with few ridges in its bark (Rechinger 1982), is distributed exclusively in the Hyrcanian Forest with other relict tree species, such as *Alnus glutinosa*, *Pterocarya fraxinifolia*, and *Quercus castaneifolia*. Because of the many morphological similarities between these two species, the taxonomic status of *P. caspica* is disputed. In fact, some botanists argue that *P. caspica* and *P. alba* belong to the same species (Rechinger 1982). When morphological similarities prevent accurate plant identification (Brinegar 2009), several molecular markers have been applied to differentiate the species (Khasa et al. 2003; Cervera et al. 2004; Wan et al. 2013).

The *trnL-trnF* (GAA) in the chloroplast genome is suitable for accurate identification of species at the intraspecific level (Kojoma et al. 2002; Yi et al. 2004; Holt et al. 2004). The length of these regions is another advantage as they are usually shorter than 700 bp (based upon most species examined to date) and can thus be directly amplified and sequenced (Tsai et al. 2006).

The *trnH-psbA* intergenic spacer is another sequence candidate for resolving lower level relationships (Nitta and O'Grady 2008; Akbarzadeh Roshan et al. 2013). These two regions can be amplified by universal primers (Taberlet et al. 1991; Pirie et al. 2007); hence, they have been the most widely used for phylogenetic studies in plants (Quandt et al. 2004). Conflicts between topologies have often been discovered from different plastid and nuclear genomes. The internal transcribed spacer (ITS) genomes have been interpreted in different ways, such as lineage sorting or reticulation (Wendel and Doyle 1998). ITS regions of nuclear ribosomal DNA have been frequently used for phylogenetic analysis of plants at lower taxonomic levels because of their universality, easy amplification, biparental inheritance and intragenomic uniformity (Fukuda et al. 2011; Yousefzadeh et al. 2012; Tang et al. 2015). Therefore, we used the ITS regions of nuclear ribosomal DNA to trace potential hybridizations events within a population of *P. caspica* by comparing bootstrap support for lineages resolved by independent topologies and combined trees.

The main objectives of this study were (1) to differentiate the two closely related species *P. caspica* and *P. alba* (section *Leuce*) and (2) to place *P. caspica* preliminarily within the genus *Populus* using sequence data from the plastid and nuclear genome.

Materials and methods

Plant material, DNA extraction, PCR and sequencing

Leaves were collected from six small populations of *P. caspica* along an east to west gradient of the Hyrcanian Forest, northern Iran (Appendix 1 in Electronic Supplementary Material), stored on ice and then brought to the lab and frozen in liquid nitrogen and milled to a fine powder. Total DNA was extracted from the ground powder using a combination of the cetyltrimethylammonium bromide (CTAB) and SDS-potassium acetate (Colagar et al. 2010). Before PCR, the DNA extract was electrophoresed in 1% agarose. The cpDNA noncoding regions *trnL-F* and *trnH-psbA* intergenic spacer (IGS) were amplified by PCR using the conditions described by Yousefzadeh et al. (2014) and specific primers UEUFF (5′-GGTTCAAGTCCCTCTATC CC-3′) as the direct primer and UEUFR (5′ATTTGAA CTGGTGACACGAG-3′) as the reverse primer.

The *trnH-psbA* region was amplified with forward primer *trn*H-GUG (5′-CGCGCATGGTGGATTCACAATCC-3′) and reverse primer *psb*A (5′-GTTATGCATGAACG-TAATGCTC-3′). The ITS region was amplified using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) as the direct primer and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as the reverse primer, which was designed by Taberlet et al. (1991) as a universal primer; the PCR conditions that were used were described by Yousefzadeh et al. (2012).

PCR products were separated by electrophoresis in 1% agarose. The *Taq*-PCR products were direct-sequenced by MWG DNA sequencing services (MWG Co., Ebersberg, Germany). The gel electrophoresis and staining were performed by Green and Sambrook (2012) methods.

Phylogenetic analysis

Electropherograms of the *trnL-F* sequences were further checked by eye using the Chromas software program version 2.33. The boundaries of sequences were determined

via comparison with samples of *Populus* in searches by Hamzeh and Dayanandan (2004) for *trnL-F* and NCBI sequence data for *trnH-psbA* and ITS. Multiple sequence alignment was performed using ClustalW2 in MEGA 6 (Tamura et al. 2013).

The genetic distances among the species were calculated according to Nei (1987), and phylogenetic inferences were made via maximum parsimony (MP). Bootstrap analysis with a fast heuristic search based on 1000 replicates was performed to assess the robustness of the branches. The cutoff value of the consensus tree was 50%, with nodes with bootstrap values of 50% considered well supported. Additionally, the phylogenetic trees were rooted using *Salix* species as outgroups.

The phylogenetic network was constructed using the median-joining algorithm with the set of SNPs identified in the ITS and *trnH-psbA* intergenic spacer. For the median-joining (MJ) network analysis, all variable characters of the complete alignment were entered into the program package PopART (Leigh and Bryant 2014; software available at: http://popart.otago.ac.nz).

Results

Characteristics of *trnL-F, trnH-psbA* and ITS

A summary of characteristics from the data matrix used in the phylogenetic analyses is provided in Table 1. The entire length of the ITS1-5.8S-ITS2 region was 569 bp for *P. caspica*; ITS1, 5.8 S and ITS2 length were 193, 163 and 214 bp, respectively. The 5.8S gene was not heterogeneous in size (156 bp). Conversely, with flowering plants, the ITS2 region had more variable sites in comparison to the ITS1 region; the *trnH-psbA* intergenic spacer ranged from 288 to 301 bp for all the two *Populus* species. Among the 288 positions of *trnH-psbA*, there were 17 parsimony positions for *P. caspica*, whereas only three sites were observed for NCBI under the study species. The *trnL-F* fragments and sequencing analysis revealed that *trnL-F* of *P. caspica* and *P. alba* was 315 bp long; 28% of total nucleotides among all samples were T (U), 15.4% were C, 38.9% A and 15.4 G (Table 1).

Phylogenetic and network analysis

The topology-based MP analyses of all three (ITS, *trnH-psbA, trnL-F*) regions are presented in Figs. 1 and 2. Based on the ITS analysis, the *Populus* species is divided into four clades. *P. caspica* taxa comprised three distinct clades with SC2, SA1 and *P. alba* (JQ898650) located in subclade 5 of clade 2 (Fig. 1), while that of *P. alba*, *P. tremula* × *P. alba*, *P. davidiana* and *P. hopeiensis* are also in this clade. Based on the phylogenetic reconstruction of the two cpDNA regions, *P. caspica* formed a separate clade from the other *Populus* samples, except for SA1 taxa in *trnH-psbA*, which were located in subclade 5 of clade 1 (Fig. 2). Additionally, phylogenetic analysis of the cpDNA regions showed differences between *P. caspica* native to the Hyrcanian region and *P. alba* collected in Canada.

The minimum and maximum pairwise genetic distance, known as the Kimura-2-parameter distance (Kimura 1980), based on ITS sequence data from all taxa in this study in comparison to NCBI GenBank taxa, was assessed (Table 2). The results showed that all the Hyrcanian taxa (SA1, NY3, G2, N1 and SC1) were at a maximum distance from *P. euphratica* (JQ898635) species. However, the distance of these taxa from *P. alba* (JQ898650) varied from zero (SA1, SC2) to 0.005, 0.007 and 0.065 for N1, G2 and NY3, respectively. Additionally, Hyrcanian taxa showed a maximum distance with *P. deltoides* (AY757053) based on

Table 1 Characteristics of the ITS and *trn*H-*psb*A sequences

cpDNA or ITS regions	*Populus caspica*					Genbank *Populus* and Hamzeh's data*				
	*trn*L-*F*	*trn*H-*psb*A	ITS			*trn*L-*F*	*trn*H-*psb*A	ITS		
			ITS1	5.8S	ITS2			ITS1	5.8S	ITS2
A (%)	38.9	37.6	15.8	22.7	12.7	38.9	37.2	15.6	22.7	12.1
C (%)	15.4	13.2	34	26.9	36.6	30.3	17.6	34.8	26.7	38.1
G (%)	17.7	12.2	35.1	28.8	32.6	14	11.5	35.1	28.8	32.2
T (U; %)	28.0	34.3	15.1	21.6	18.2	16.9	33.7	14.5	21.7	17.6
Length (bp)	315	288	192	163	214	335	288–301	193–195	163	212
Conservation site	303	207	188	163	183	296	284	180	162	194
Variable site	10	80	4	0	30	13	10	12	0	18
Parsimony site	1	17	3	0	1	7	3	8	0	10

*Dr. Mona Hamzeh (Department of Biology, Concordia University, Canada)

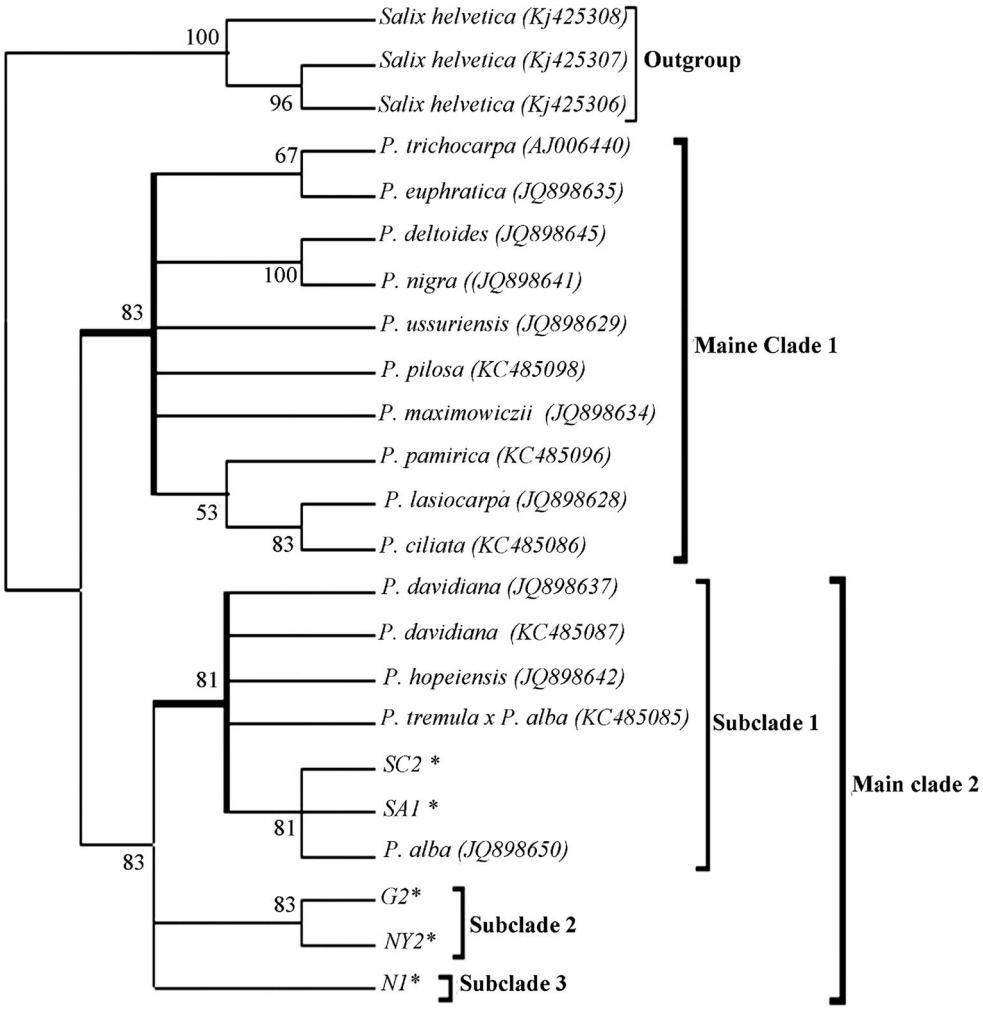

Fig. 1 Taxonomic position of *P. caspica* within the genus *Populus* based on the analysis of the nuclear DNA sequences (ITS-5.8S-ITS2) from this study with selected species from the NCBI gene bank: Maximum parsimony consensus original tree (> 50%); Bootstrap 500; *Salix* sp. as an outgroup. Asterisk indicates samples from the Hyrcanian region

cpDNA and were differentiated from *P. alba* with a distance of 0.046, except for SA1 at 0.047 (Table 3).

According to the network analysis of cpDNA (*trnH-psbA* and *trnL-F*), all Hyrcanian taxa originated from *P. deltoides* as haplogroup 1, except one taxa that originated from *P. alba*, as haplogroup 2 (Fig. 3a). Additionally, two ribotypes were identified according to the ITS analysis, which originated from *P. tremula* × *P. alba*. These samples are N1, G2, and NY3 (ribotype 1) and SC2, SA1 (ribotype 2), as shown in Fig. 3b. Amplification of the entire ITS yielded two bands of approximately 700 and 500 bp in some samples of *P. caspica.*

Discussion

Despite recent advances in the biology of *Populus* species, the relationships between species and taxonomic status of purported hybrids have remained controversial. For example, Gong (2004) classified *P. pseudoglauca* in the section *Tacamahaca*, while Ding and Fang (1993) proposed that this species belongs to the section *Leucoides* (Wan et al. 2013). Traditional classification studies are based only on the morphological characteristics without genetic identification, although morphological characteristics are easily influenced by environmental conditions. It is very difficult to precisely identify some species or varieties of *Populus* based strictly on morphology, particularly for similar species (Wan et al. 2013). The present study using chloroplast and nuclear nucleotide sequences to assess genetic differentiation between *P. caspica*, which is native to the Hyrcanian forest, and *P. alba*, revealed (1) significant topological incongruence, (2) significantly different data partitions between the cpDNA regions, (3) geographical differentiation among *P. caspica* populations, and (4) two variants of ITS in some *P. caspica* individuals.

This study showed that *P. caspica* sequences are different from those of *P. alba* (from studies by Hamzeh and Dayanandan 2004 and from NCBI GenBank). However, our results also showed that the sequences vary among the *P. caspica* populations. Sytsma and Schaal (1985)

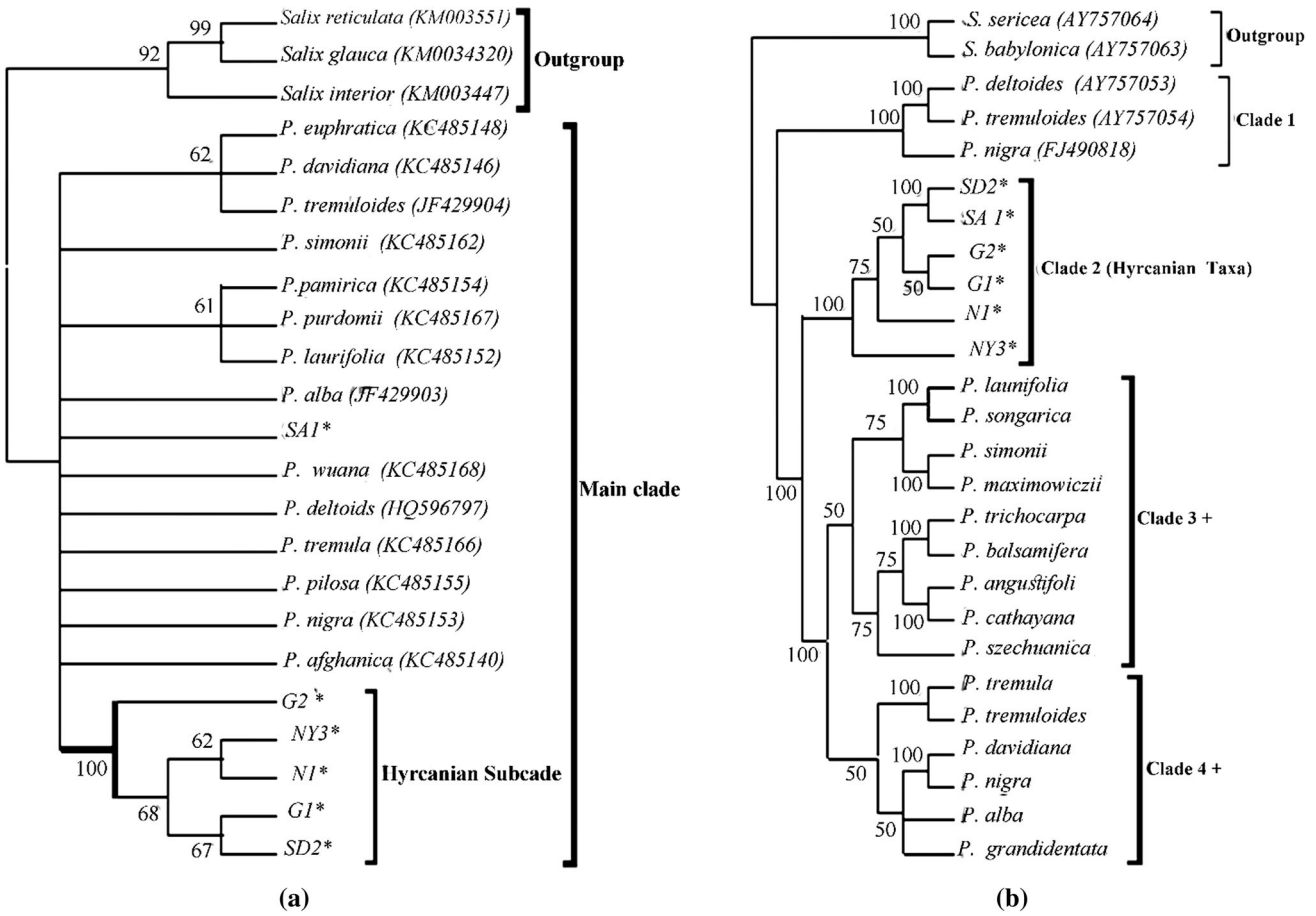

Fig. 2 Taxonomic position of *P. caspica* within the genus *Populus* based on the cpDNA analysis: **a** *trnH-psbA*, **b** *trnL-F*. Maximum parsimony consensus original tree (> 50%), Bootstrap 500; Asterisk and plus symbols indicate the Hyrcanian samples and taxa obtained from Dr. Mona Hamzeh (Department of Biology, Concordia University, Canada), respectively

attributed variation in rDNA to genetic drift arising from spatial and genetic isolation between populations.

In recent years, many *P. caspica* habitats have been destroyed due to urbanization and changes in land use. Habitat fragmentation reduced extensive gene flow among populations and caused high genetic differentiation. Geographical differentiation of variants was observed within a population of *Clematis fremontii*, indicating population subdivisions consistent with limited gene flow within the population (Learn and Schaal 1987). Differentiation of rDNA among populations of both *Picea rubens* and *P. mariana* was attributed to genetic drift or forces related to ecogeographical selection (Bobola et al. 1992). Directional selection was responsible for substantial change in allelic frequencies in a population of barley over 53 generations (Saghai-Maroof et al. 1984). Genetic differentiation between populations was also observed in *Phlox* because of limited gene flow between populations (Schaal et al. 1987).

In this study, we observed conflicts between ITS and plastid topologies. The conflicts between topologies from different genomes can be explained by lineage sorting or reticulation (Wendel and Doyle 1998), existence of past hybridization events and ITS paralogy (Catalan et al. 2004).

The conflicts between ITS and cpDNA topologies might be explained as convergent evolution, lineage sorting, or reticulate evolution (Raamsdonk et al. 1997; Degnan and Rosenberg 2009; Pelser et al. 2010; Feng et al. 2013; Leskinen and Alström-Rapaport 1999). In this work, a topological incongruence was noted for the two cpDNA regions, with displacement of the SA1. For the ITS data, SA1 and SC2 clustered with *P. alba*, but the other three samples were grouped in a separate clade (Clade 2). Different positions of SA1 on the cpDNA phylogenies indicate that cpDNA heterologous recombination occurred from more or less ancient hybridization, which has also been reported for the cpDNA genome of *Picea* (Bouillé et al. 2011) and lodgepole pine (Marshall et al. 2001). However, the nrITS phylogenies support the recent hybridization between two species.

Additionally, within some individuals of *P. caspica*, two variants of ITS were observed. Similar patterns of multiple

Table 2 Minimum and maximum pairwise genetic distance (Kimura-2-parameters distance) among analyzed *Populus* species based on the nuclear (ITS-5.8S-ITS2) DNA

	N1	SC2	SA1	G2	NY3	6*	7*	8*	9*	10*
N1	0.000									
SC2	0.065	0.000								
SA1	0.065	0.000	0.000							
G2	0.061	0.007	0.007	0.000						
NY3	0.059	0.005	0.005	0.002	0.000					
P. tremula (KC485085)	0.063	0.002	0.002	0.005	0.004	0.000				
P. trichocarpa (AJ006440)	0.087	0.028	0.028	0.029	0.028	0.026	0.000			
P. alba (JQ898650)	0.065	0.000	0.000	0.007	0.005	0.002	0.028	0.000		
P. deltoids (JQ898645)	0.085	0.026	0.026	0.028	0.026	0.024	0.013	0.026	0.000	
P. hopeiensis (JQ898642)	0.063	0.002	0.002	0.005	0.004	0.000	0.026	0.002	0.024	0.000
P. nigra (JQ898641)	0.085	0.026	0.026	0.028	0.026	0.024	0.013	0.026	0.000	0.024
P. davidiana (JQ898637)	0.063	0.002	0.002	0.005	0.004	0.000	0.026	0.002	0.024	0.000
P. euphratica (JQ898635)	0.103	0.041	0.041	0.043	0.041	0.039	0.020	0.041	0.029	0.039
P. maximowiczii (JQ898634)	0.078	0.020	0.020	0.022	0.020	0.018	0.009	0.020	0.013	0.018
P. ussuriensis (JQ898629)	0.078	0.020	0.020	0.022	0.020	0.018	0.009	0.020	0.013	0.018
P. lasiocarpa (JQ898628)	0.080	0.022	0.022	0.024	0.022	0.020	0.018	0.022	0.016	0.020
P. pilosa (KC485098)	0.078	0.020	0.020	0.022	0.020	0.018	0.009	0.020	0.013	0.018
P. pamirica (KC485096)	0.080	0.022	0.022	0.024	0.022	0.020	0.014	0.022	0.016	0.020
P. davidiana (KC485087)	0.063	0.002	0.002	0.005	0.004	0.000	0.026	0.002	0.024	0.000
P. ciliata_voucher (KC485086)	0.078	0.020	0.020	0.022	0.020	0.018	0.016	0.020	0.014	0.018

	11*	12*	13*	14*	15*	16*	17*	18*	19*
N1									
SC2									
SA1									
G2									
NY3									
P. tremula (KC485085)									
P. trichocarpa (AJ006440)									
P. alba (JQ898650)									
P. deltoids (JQ898645)									
P. hopeiensis (JQ898642)									
P. nigra (JQ898641)	0.000								
P. davidiana (JQ898637)	0.024	0.000							
P. euphratica (JQ898635)	0.029	0.039	0.000						
P. maximowiczii (JQ898634)	0.013	0.018	0.026	0.000					
P. ussuriensis (JQ898629)	0.013	0.018	0.026	0.000	0. 000				
P. lasiocarpa (JQ898628)	0.016	0.020	0.028	0.009	0.009	0.000			
P. pilosa (KC485098)	0.013	0.018	0.026	0.000	0.000	0.009	0.000		
P. pamirica (KC485096)	0.016	0.020	0.028	0.005	0.005	0.007	0.005	0.000	
P. davidiana (KC485087)	0.024	0.000	0.039	0.018	0.018	0.020	0.018	0.020	0.000
P. ciliata_voucher (KC485086)	0.014	0.018	0.029	0.007	0.007	0.002	0.007	0.005	0.018

*6: *P. tremula* (KC485085); 7: *P. trichocarpa* (AJ006440); 8: *P. alba* (JQ898650): 9: *P. deltoids* (JQ898645); 10: *P. hopeiensis* (JQ898642); 11: *P. nigra* (JQ898641); 12: *P. davidiana* (JQ898637); 13: *P. euphratica* (JQ898635); 14: *P. maximowiczii* (JQ898634); 15: *P. ussuriensis* (JQ898629); 16: *P. lasiocarpa* (JQ898628); 17: *P. pilosa* (KC485098); 18: *P. pamirica* (KC485096); 19: *P. davidiana* (KC485087); 20: *P*

Table 3 Minimum and maximum pairwise genetic distance (Kimura-2-parameters distance) among analyzed *Populus* species based on cpDNA*

	1	2	3	4	5	6	7	8	9	10
P. deltoides (AY757053)										
P. nigra (FJ490818)	0.008									
P. tremuloides (AY757054)	0.016	0.008								
*P. laurifolia**	0.812	0.784	0.790							
*P. songarica**	0.812	0.784	0.790	0.000						
*P. simonii**	0.812	0.784	0.790	0.000	0.000					
*P. maximowiczii**	0.812	0.784	0.790	0.000	0.000	0.000				
*P. davidiana**	0.842	0.812	0.818	0.008	0.008	0.008	0.008			
*P. grandidentata**	0.812	0.784	0.790	0.000	0.000	0.000	0.000	0.008		
*P. tremula**	0.812	0.784	0.790	0.000	0.000	0.000	0.000	0.008	0.000	
*P. alba**	0.812	0.784	0.790	0.000	0.000	0.000	0.000	0.008	0.000	0.000
*P. tremuloides**	0.812	0.784	0.790	0.000	0.000	0.000	0.000	0.008	0.000	0.000
*P. nigra**	0.842	0.812	0.818	0.008	0.008	0.008	0.008	0.000	0.008	0.008
*P. trichocarpa**	0.790	0.763	0.768	0.008	0.008	0.008	0.008	0.016	0.008	0.008
*P. balsamifera**	0.790	0.763	0.768	0.008	0.008	0.008	0.008	0.016	0.008	0.008
*P. angustifoli**	0.842	0.812	0.818	0.008	0.008	0.008	0.008	0.000	0.008	0.008
*P. cathayana**	0.842	0.812	0.818	0.008	0.008	0.008	0.008	0.000	0.008	0.008
*P. szechuanica**	0.812	0.784	0.790	0.000	0.000	0.000	0.000	0.008	0.000	0.000
SD2 (this study)	0.558	0.540	0.524	0.461	0.461	0.461	0.461	0.478	0.461	0.461
G2 (this study)	0.558	0.540	0.524	0.461	0.461	0.461	0.461	0.478	0.461	0.461
SA1 (this study)	0.542	0.524	0.508	0.476	0.476	0.476	0.476	0.493	0.476	0.476
NY3 (this study)	0.558	0.540	0.524	0.461	0.461	0.461	0.461	0.478	0.461	0.461
N1 (this study)	0.558	0.540	0.524	0.461	0.461	0.461	0.461	0.478	0.461	0.461
G1 (this study)	0.558	0.540	0.524	0.461	0.461	0.461	0.461	0.478	0.461	0.461

	11	12	13	14	15	16	17	18	SD2	G2	SA1	NY3	N1
P. deltoides (AY757053)													
P. nigra (FJ490818)													
P. tremuloides (AY757054)													
*P. laurifolia**													
*P. songarica**													
*P. simonii**													
*P. maximowiczii**													
*P. davidiana**													
*P. grandidentata**													
*P. tremula**													
*P. alba**													
*P. tremuloides**	0.000												
*P. nigra**	0.008	0.008											
*P. trichocarpa**	0.008	0.008	0.016										
*P. balsamifera**	0.008	0.008	0.016	0.000									
*P. angustifoli**	0.008	0.008	0.000	0.016	0.016								
*P. cathayana**	0.008	0.008	0.000	0.016	0.016	0.000							
*P. szechuanica**	0.000	0.000	0.008	0.008	0.008	0.008	0.008						
SD2 (this study)	0.461	0.461	0.478	0.476	0.476	0.478	0.478	0.461					
G2 (this study)	0.461	0.461	0.478	0.476	0.476	0.478	0.478	0.461	0.000				
SA1 (this study)	0.476	0.476	0.493	0.491	0.491	0.493	0.493	0.476	0.008	0.008			
NY3 (this study)	0.461	0.461	0.478	0.476	0.476	0.478	0.478	0.461	0.000	0.000	0.008		

Table 3 continued

	11	12	13	14	15	16	17	18	SD2	G2	SA1	NY3	N1
N1 (this study)	0.461	0.461	0.478	0.476	0.476	0.478	0.478	0.461	0.000	0.000	0.008	0.000	
G1 (this study)	0.461	0.461	0.478	0.476	0.476	0.478	0.478	0.461	0.000	0.000	0.008	0.000	0.000

**trnL-F* sequences of these taxa obtained from Dr. Mona Hamzeh (Department of Biology, Concordia University, Canada); 1: *P. deltoides* (AY757053); 2: *P. nigra* (FJ490818); 3: *P. tremuloides* (AY757054); 4; *P. laurifolia*; 5: *P. songarica*; 6: *P. simonii*; 7: *P. maximowiczii*; 8: *P. davidiana*: 9: *P. grandidentata*; 10: *P. tremula*; 11: *P. alba*; 12: *P. tremuloides*; 13; *P. nigra*; 14: *P. trichocarpa*; 15: *P. balsamifera*; 16: *P. angustifoli*; 17: *P. cathayana*; 18: *P. szechuanica*

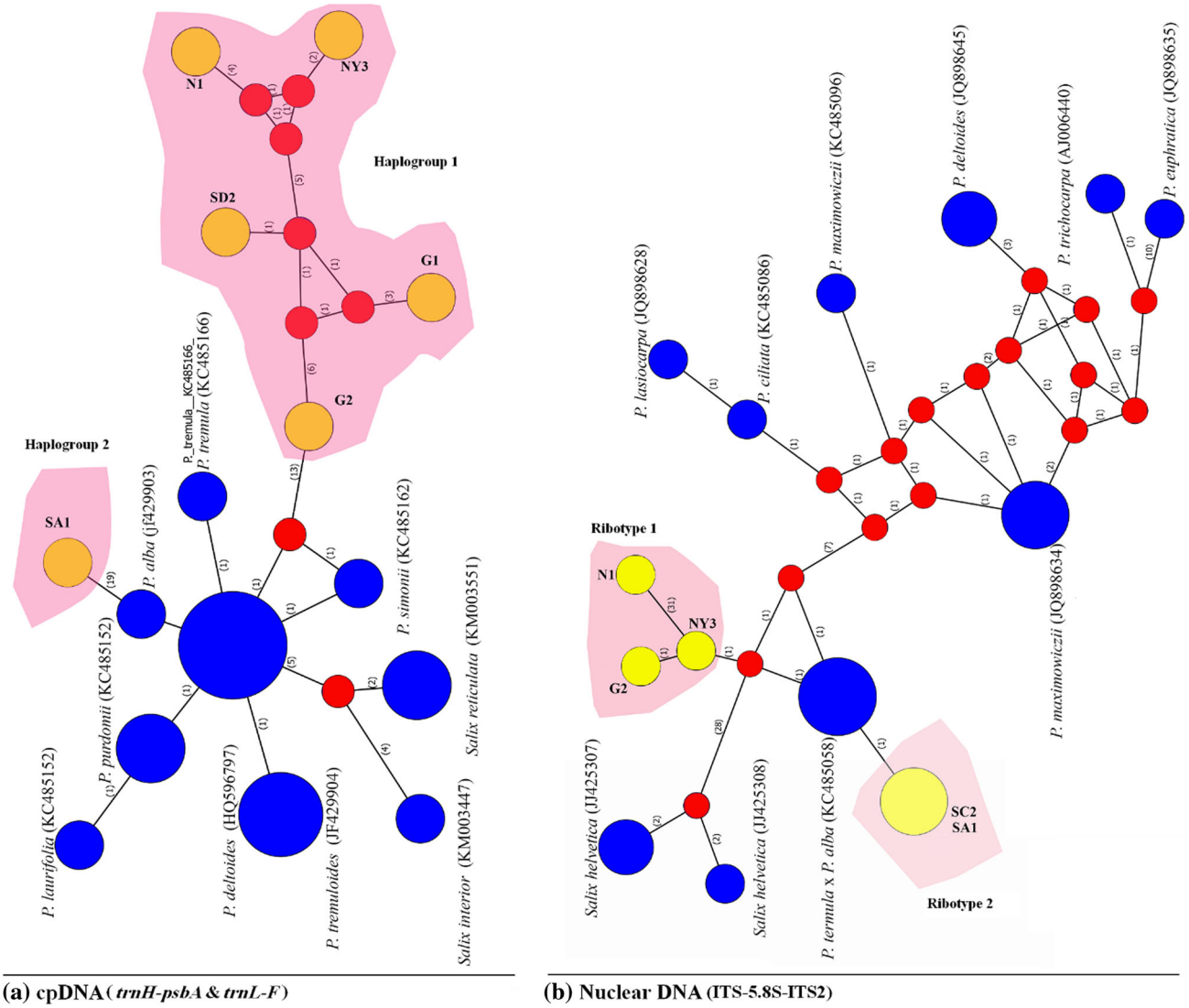

Fig. 3 Phylogenetic network combining chloroplast and nuclear DNA data within the genus *Populus*: **a** cpDNA (*trnH-psbA* and *trnL-F*), **b** nuclear DNA (ITS-5.8S-ITS2); Blue, red and yellow circles are proportional to the number of species belonging to the same SNP, median vector and the Hyrcanian samples, respectively

rDNA length variants within individuals have been observed in other long-lived outbreeding species, such as wind-pollinated oaks (Bellarosa et al. 1990) and insect-pollinated rubber trees (Besse et al. 1993).

Differences in life history traits, breeding systems and the level of gene flow are the main determinant factors of extensive outcrossing of individuals within and between populations. Therefore, the level of gene flow in this

species with a foreign species is sufficient to prevent both the homogenization of rDNA variants within individuals and the divergence of rDNA between populations (Mclain et al. 1995; Keller et al. 2008).

Numerous examples of natural or spontaneous hybridization in *Populus* have been documented or proposed (Barnes and Pregitzer 1985; Smith and Sytsma 1990). Extensive planting of exotic poplars, such as *P.* × *euramericana* and *P. termuloides*, in the Hyrcanian forest increase a risk of hybridization and introgression. The possibility of hybridization between *P. alba* and *P. tremuloides* and between *P. alba* and *P. tremula* are confirmed and are called *Populus* × *heimburgeri* Bovin and *Populus* × *canescens*, respectively. In this case, gene exchange (hybridization and introgression) between *P. caspica* and exotic species considered as genetic pollutants is of great concern for forest trees because they can have large effects on ecosystem processes and biological diversity (Nishimoto et al. 2003; DiFazio et al. 2004). Poplar plantations with foreign species, such as *P. euroamericana*, are a threat to the diversity and regeneration of native indigenous poplars. For example, in Europe, the European black poplar is threatened by the euramerican *P.* × *canadensis* Moench. and interamerican *P.* × *generosa* Henry hybrids as well as by *P. nigra* varieties, such as the male Lombardy poplar, *Populus* nigra cv. *Italica* Duroi (Heinze 1998; Lefèvre et al. 2001). Habitat fragmentation, human activities for reduction of plains area and widespread cultivation of exotic species are three main reasons for *P. caspica* being on the verge of extinction. Therefore, *P. caspica* is the only poplar forest species in the Hyrcanian forest faced with a large threat due to agriculture and urbanization of floodplain areas and hybridization with cultivated poplar that threaten the survival of the species.

Conclusions

Populus caspica is an indicator and endemic tree species growing in Hyrcanian forests together with *Parrotia persica*, *Ulmus carpinifolia*, *Alnus glutinosa*, *Quercus castaneifolia*, *Pterocarya fraxinifolia*, and others. Habitat fragmentation, human activities for the reduction of the plains area and widespread cultivation of exotic species are three main reasons for *P. caspica* being on the verge extinction. High differentiation of *P. caspica* from the other *Populus* species confirmed the special taxonomic status of this species in the genus. This study has also demonstrated the presence of hybrids between cultivated *Populus* with *P. caspica* in the Hyrcanian forest. Additionally, our study showed the presence of two ITS copies in some individuals, consistent with observations of spontaneous hybridization among these species in nature and in cultivation (Hamzeh and Dayanandan 2004). For example, hybrid *P.* × *euramericana* is a spontaneous hybrid between European *P. nigra* and North American *P. deltoides* (Lefèvre et al. 2001; Hamzeh et al. 2006).

Hybridization could occur between *P. caspica* and *P. deltoides* and or *P.* × *euramericana* because these tree species are cultivated in the Hyrcanian region. Thus, inter- and intra-sectional hybridization between *P. caspica* with other species is a major cause of the disagreement on the total number of poplar species and their classification.

However, due to strong disturbance and fragmentation, conservation of large parts of natural distribution areas of *P. caspica* might not be possible, so protection of selected stands of this species is highly recommended (in situ conservation) to provide stock material for further breeding and maintaining a large gene pool that will evolve over time in response to environmental changes. Further studies on intra-genomic rDNA variation in *Populus* species might shed more light on the fundamental mechanisms of concerted evolution and speciation and also environmental rDNA sequence data in plants.

This study is the first report on the phylogenetic relationship of *P. caspica* with other *Populus* species. However, using morphological data and other molecular markers, such as genus-specific microsatellite markers, and sharing voucher specimens with other *Populus* genetic researchers could be useful for more accurate conclusions.

Acknowledgements We are tremendously grateful to Dr. Mona Hamzeh, Department of Biology, Concordia University, for providing of some Canadian samples sequences. Also, we thank Fatemeh Mostajeran and Hamid Bina, Tarbiat Modares University, Noor, Mazandaran, Iran, for help with data collections.

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