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Genetic diversity in some euphrates poplar (*Populus euphratica* O.) ecotypes in Iran using microsatellites (SSRs) markers

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Abstract

Euphrates poplar *(Populus euphratica* Oliv.) is a unique woody species which is naturally distributed in desert areas of some parts of Asia and Africa. Because of its outstanding features, it is a model plant to study environment stress tolerance. This research was conducted to evaluate the genetic variation in 12 ecotypes of *P. euphratica* in Iran through 10 simple sequence repeats (SSRs) primers from 2015 to 2016. The average numbers of alleles observed in each ecotype was 6.43 and average numbers of effective alleles was 5.58. The average of observed heterozygosis was 0.65 and average of expected heterozygosis was 0.80. The ecotypes were complying with Hardy-Weinberg's equilibrium in all loci, except Marand ecotype for two of the ten primers that showed deviation of the balance ($p<0.05$). The Shannon information index was 1.75. Analysis of molecular variance (AMOVA) showed that 3% of molecular variance belongs to intra-population and 97% belongs to inter-population. The PCA showed six principal components covered 22.86% of the total variance. Clustering analysis of ecotypes through genetic distance, the examined ecotypes were divided into six groups, while the geographic distance did not have any significant effect on genetic differences. Overall, the results indicated that *P. euphratica* stands covered a vast area of Iran in the past, and probably had not been fragmented; it seems vast areas of Iran are potentially ready for revival of *P. euphratica* forests.

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Introduction

Conservation of genetic resources is one of the fundamental steps for the preservation, improvement, and development of ecosystems in each region. Therefore, the preservation of plant and animal germplasm is vital because of its presence in the region for centuries and thousands of years and its adaptation to different soil and climatic stresses.

Populus euphratica Oliv. is one of the native plants which has grown for thousands of years in many parts of Iran and some parts of the world. In some cases it has been consistently present in limited biological conditions. This species is naturally spread in large areas of Asia, Europe, and Africa. Its presence has also been reported historically in vast arid and semi-arid areas of Iran. Tolerance to drought and soil salinity and wind erosion in arid areas are important characteristics of this species. Its presence and propagation in different ecological conditions may have led to the genetic diversity of *P. euphratica* ecotypes. Generally, Poplar species are fast-growing with high productivity, easy replication and multiple applications; therefore they are very important for the development of afforestation and plantation in different ecological areas. The small genome and the ease of tissue culture have introduced them as a model system to evaluate the genetic and molecular biology in the woody plants (Lin *et al.*, 2006; Tuskan *et al.*, 2006).

Generally, there is some evidence showing the high variability of inter-population using SSR marker (Wang *et al.*, 2011). Pascal *et al.*, (2009) evaluated 18 primer pairs and observed 2 to 17 alleles (average 4.6) for 13 loci. Wu, *et al.* (2008) observed 5 to 9 alleles in 12 loci and ranged from 0.32 to 0.48 and from 0.53 to 0.67 for observed and expected heterozygosis, respectively. None of the loci had significant deviation from Hardy-Weinberg's equilibrium; however, these markers were suitable for the study of *P. euphratica* population genetic structure. Saito *et al.*, (2002) in the study of natural *P. euphratica* populations in northwestern China observed geographical distance had no significant effect on genetic differences. The objectives of this study were to evaluate and compare genetic variation among and within *P. euphratica* populations in some natural habitats of Iran.

Materials and methods

Sampling strategy

In this study, leaf samples of *P. euphratica* were collected from 12 regions of Iran. Table 1 shows the locations and properties of the collection areas and Fig.1 illustrates the collection areas in Iran's map. Several attributes were considered for selection of theses natural habitats, including climate variability, different soil and water conditions, and a wide geographical scope.

Table 1. Collection areas information.

Name of region	Province	Symbol	longitude	Latitude	Elevation (m)	Annual temperature (°C)	Annual precipitation (mm)
Jolfa	Azerbaijan	E1	38 57 N	45 41 E	0703	14.4	179.8
Marand	Azerbaijan	E2	38 31 N	45 24 E	1077	12.3	342.2
Maranjab	Esfahan	E3	34 13 N	51 40 E	0930	18.8	138.4
Manjil	Gilan	E4	36 15 N	49 26 E	0330	17.3	196.4
Dashlibrun	Golestan	E5	37 46 N	54 54 E	0037	17.1	201.9
Sarakhs	Khorasan	E6	36 18 N	61 09 E	0303	17.6	203.3
Dezful	Khuzestan	E7	32 14 N	48 20 E	0063	24.0	444.3
Hamidieh	Khuzestan	E8	31 31 N	48 28 E	0023	24.2	194.5
Mahalat	Markazi	E9	34 00 N	50 33 E	1850	12.8	294.2
Masumieh	Qom	E10	34 43 N	50 52 E	0910	18.7	146.1
Gilvan	Zanjan	E11	36 46 N	49 26 E	0376	17.3	196.4
Mahneshan	Zanjan	E12	36 46 N	47 43 E	1706	14.6	207.0

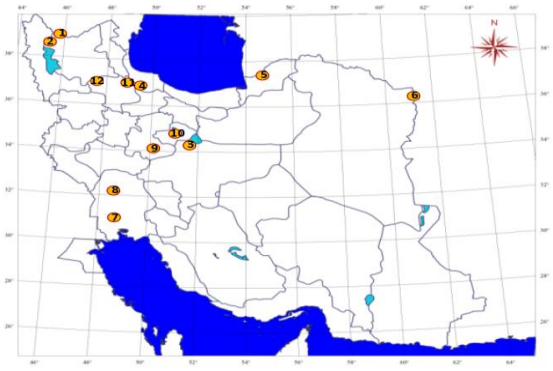

Fig. 1. Collection areas in Iran's map.

In order to study the molecular diversity, leaf samples were taken from five trees with the greatest possible distance in each habitat.

To evaluate the variation within and among populations of 12 ecotypes, 10 primer pairs of SSR markers were used. The selected Primer sequences used for the amplification of DNA template are shown in Table 2.

Table 2. Characteristics of the 10 microsatellite l oci.

NO.	*Locus*	*Repeat*	*Primer sequence (5′→3′)*	Size Range (bp)	Nucleotide Numbers	Annealing Temperature (°C)	Reference
1	*Pe7*	*(TG)10*	*ACTATGGAGCACGCAAAATGCCAAG* *TAGTTCCTGCTCTGCTTATCGTC*	170-228	25 23	65.8 62.9	Wu *et al.* (2008)
2	*Pe8*	*TG)12-(GA)11*	*AACACGGAAGCAAGAAAAAATGAAG* *CACACATTCCACCCTCCACACCACT*	146-197	25 25	60.9 69.1	Wu *et al.* (2008)
3	*Pe9*	*(GT)26*	*TTCCAAATAAGCTTATTGTAAACCC* *CATTTCACAGTGGGACCAATTCACA*	168-218	25 25	59.2 64.1	Wu *et al.* (2008)
4	*Pe13*	*(CT)6-(GT)*	*TTCAACTTGACTAGTTGTAACTCTC* *CACTTTCCCAGCTATCCCTTTCTAA*	121-137	25 25	60.9 64.1	Wu *et al.* (2008)
5	*Pe14_2*	*(CT)11*	*CCTTCGAAATGGGAGATCTGT* *CACCACAACAGCGTACAGAAA*	146-178	21 21	59.5 59.5	Xu *et al.* (2013)
6	*Pe16*	*(AC)17*	*CACCTATAAGGAAAATCACAAGTGT* *CAGATACTAATGAAGAGATCTTCGG*	349-281	25 25	60.9 62.5	Wu *et al.* (2008)
7	*Pe17_2*	*(AC)13*	*ACGACGACTGCTTCGAGTTT* *TGGTGGTAGGAGGAAGAAGAA*	180-211	20 21	58.4 59.5	Xu *et al.* (2013)
8	U15717	(GA)11	TCCCTTCTCTTCCGTTCTCA CCTCCGATTAGGGCTTTCTC	124-240	20 20	58.4 60.5	Xu *et al.* (2013)
9	U47561	(AT)15	TGAAGATGGTCCACAGCAAG GGTGGTGTACAGGGCAGTTT	91-110	20 20	58.4 60.5	Xu *et al.* (2013)
10	ORPM 030	(TC)9	ATGTCCACACCCAGATGACA CCGGCTTCATTAAGAGTTGG	207-229	20 20	58.4 58.4	Pascal *et al.* (2009)

DNA isolation

Genomic Deoxyribonucleic acid **(**DNA) was isolated from each individual using the hexadecetyltrim-ethylammonium bromide (CTAB) method (Murray and Thompson, 1980) with some modifications. One square centimeter of leaf was cut into Fine powder in liquid nitrogen and put in a 2.0 ml microtube; 800 µL of extraction buffer was added [CTAB 20 mg/mL, EDTA 0.02 M and Tris HCl 0.1 M (pH 8.0), NaCl 1.4 M, with 0.75% b-Mercaptoethanol added immediately before use]. The microtubes were held vertically on a shaker and incubated at 65°C for 1 h. After cooling, 650 µL of chloroform/iso-amylalcohol (24:1) was added, and the resulting mixture was shaken for 10 min. An equal volume of cold isopropanol was then added, and the sample was stored at -20°C for 1 h, the upper phase was transferred to a new micro-tube. Genomic DNA was precipitated by centrifuging at 12,000 g for 10 min. After several washes with ethanol, the pellets were dried and diluted in pure water. The DNA concentration of the resulting solutions was determined using a Nano Drop 1,000 spectrophotometer (Thermo, USA), after which they were transferred to a 96-well plate and adjusted to 10–15 ng/µL.

PCR development

Each 20 µL polymerase chain reaction (PCR) mixture contained 1X PCR buffer, 3 mM $MgCl_2$, 0.5 mMdNTPs, 1 U Taq polymerase, 0.5 pmol/µL of the forward primer, 0.5 pmol/µL of the reverse primer and 3.75 ng/µL templates DNA. PCR amplifications were performed using a Thermocycler (BioradIcycler, USA) using the following thermal cycle:

Initialization at 94°C for 5 min; 35 cycles of denaturation at 94°C for 40 s, annealing at 46°C to 56°C (according to primer's annealing temperature) for 40 s and elongation at 72°C for 45 s; and a final extension at 72°C for 10 min. Approximately 0.5 µl sample of the PCR products were electrophoresis on agarose gel 3% metaphor with 80 volts for 3 hours, and then were stained with Ethidium bromide for 30 minutes, appearing bands were captured using gel-document device (Gel Doc Bio-rad, USA).

Data analysis and statistical calculations

SSR data were analyzed using Gen ALEx 6.1 software. According to this analysis features such as alleles number (Na), effective alleles number (Ne), Shannon's information index (I), observed heterozygosis (Ho), expected heterozygosis (He), coefficient of deviation from Hardy-Weinberg's equilibrium within and among populations and level of gene flow (Nm) were calculated. Molecular analyses were performed such as analysis of molecular variance (AMOVA) and principal components analysis (PCA).Cluster analysis were calculated based on Nei's genetic distance and using NTSYS 2.02e software, polymorphism information content (PIC) in loci were calculated using Arlequin 3.11 software.

Results and discussion

The results showed that there was a high polymorphic for SSR loci in *P. euphratica* ecotypes. The average number of alleles was 34 and average of allele frequencies was calculated about 0.09 in each primer pairs. The average polymorphism information content (PIC) was calculated 0.95 for 10 primer pairs. The average number of alleles calculated in this study was higher than the average number of alleles reported in previous studies for populations in China (Wang *et al.*, 2011; Xu *et al.*, 2013).

In this study on 12 ecotypes of *P. euphratica* in Iran, average numbers of alleles observed in each ecotype (Na) was 6.43 and average numbers of effective alleles (Ne) was 5.58.

The average of observed heterozygosis (Ho) was 0.65 and average of expected heterozygosis (He) was 0.80. Shannon's information index (I) in this study was lower than that reported by Wang *et al.* (2011) in northwest China. The decreased diversity is possibly due to the smaller stands in Iran compared to those in China (Table 3).

Table 3. Genetic variation within population of *P. euphratica* based on 10 SSR loci.

Ecotype	Na	Ne	I	Ho	He	F
E1	6.600	5.884	1.806	0.695	0.821	0.161
E2	4.900	4.168	1.455	0.613	0.734	0.161
E3	5.800	5.041	1.654	0.622	0.787	0.204
E4	7.400	6.493	1.922	0.715	0.840	0.149
E5	6.200	5.134	1.672	0.535	0.775	0.326
E6	6.900	5.891	1.837	0.640	0.823	0.225
E7	6.400	5.878	1.780	0.618	0.818	0.256
E8	7.400	6.562	1.936	0.735	0.845	0.129
E9	6.800	5.807	1.804	0.765	0.811	0.104
E10	6.600	5.614	1.770	0.635	0.805	0.211
E11	6.400	5.396	1.739	0.625	0.799	0.230
E12	5.800	5.027	1.610	0.645	0.762	0.168
Mean	**6.433**	**5.575**	**1.749**	**0.654**	**0.802**	**0.189**

Ne=No. of effective Allels, I=Shanon,s Information Index, Ho=Observed Heterozygosity, He=Expected Heterozygosity, F=Fixation Index.

The ecotypes were complying with Hardy-Weinberg's equilibrium in all loci, except Marand ecotype (E2) for primers (Pe7, Pe9) that showed deviation of the balance ($p<0.05$). The results of this study were in agreement with those of Saito *et al.* (2002), who reported *P. euphratica* populations were in the Hardy-Weinberg's equilibrium. The reasons for deviation of Marand (E2) ecotype from Hardy-Weinberg's equilibrium can be smaller size and purity of the stand and lack of regeneration of the trees in that site. The data analysis of molecular variance (AMOVA) showed that 3% of molecular variance belongs to intra-population and 97% belongs to inter-population (Table 4); however, the results of this study were in agreement with those of Wang *et al.* (2011). The percentage of variation among and within ecotypes are showed in Fig. 2.

Table 4. Analysis of molecular variance (AMOVA) for diversity within and among ecotypes.

Source	df	SS	MS	Est. Var.	%
Among Pops	11	164.167	14.924	0.407	3%
Within Pops	48	618.800	12.892	12.892	97%
Total	59	782.967		13.298	100%

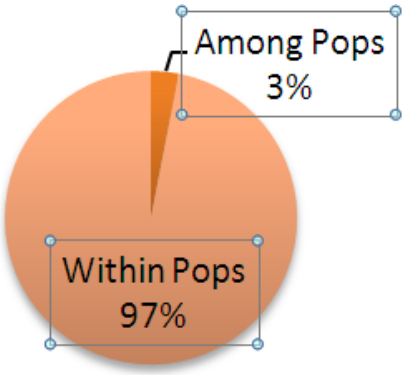

Fig. 2. Percentage of molecular variance within and among populations.

The gene flow (Nm) was estimated about 7.93; it is relatively high. This indicates the possibility of gene exchange among populations. However, the results of this study were in agreement with those of Wang *et al.* (2011) who reported the value of F_{st} from 0.025 to 0.165 and gene flow (Nm) 2.446. High gene flow among the populations indicated that there was a sort of gene exchange between them.

This confirms that probably populations have not been fragmented in the past and have become fragmented today. It is also possible that dispersion of tree seeds by wind or water may be geographically widespread and in long distances.

The PCA showed six principal components covered 22.86% of total variance. Generally genetic distance among populations was relatively low; the average genetic distance was 1.862 (Table 5). High genetic similarity has been made from the merger and the populations as a large overlap of gene flow among populations.

Table 5. Pairwise Population Matrix of Nei Genetic Distance.

pop1	pop2	pop3	pop4	pop5	pop6	pop7	pop8	pop9	pop10	pop11	pop12	
0.000												pop1
1.226	0.000											pop2
1.648	2.124	0.000										pop3
1.694	1.986	1.126	0.000									Pop4
1.840	1.924	1.664	1.335	0.000								pop5
1.608	2.137	2.568	1.624	1.475	0.000							pop6
1.916	2.200	2.114	2.151	2.522	1.943	0.000						pop7
1.387	2.330	1.823	1.873	2.140	2.208	1.313	0.000					pop8
1.573	1.554	2.320	1.865	1.553	1.309	1.814	1.681	0.000				pop9
1.795	1.969	1.617	1.641	1.713	2.090	2.015	2.214	1.451	0.000			pop10
1.395	2.069	1.488	1.498	1.538	2.584	1.925	1.445	1.540	1.330	0.000		pop11
2.038	2.183	2.615	2.362	2.078	1.888	2.034	2.168	2.202	1.784	2.653	0.000	pop12

The results showed that the genetic distance was not correlated with geographical distance; however, the result of this study was consistent with that of Wang *et al.* (2011).

Cluster analysis based on UPGMA and distance matrix (Nei and Li, 1979) was created. Accordingly, in approximately 1.50 from coefficient of genetic distance, the ecotypes were divided into 6 groups (Fig. 3).

However, the position of some ecotypes in terms of clustering was consistent with the geographical distance; but in general, clustering of more ecotypes in this study showed that geographical distance has no significant effect on genetic differences. The results of this study were in agreement with those of Saito *et al.* (2002) in evaluation of stands *P. euphratic* in Northwest China.

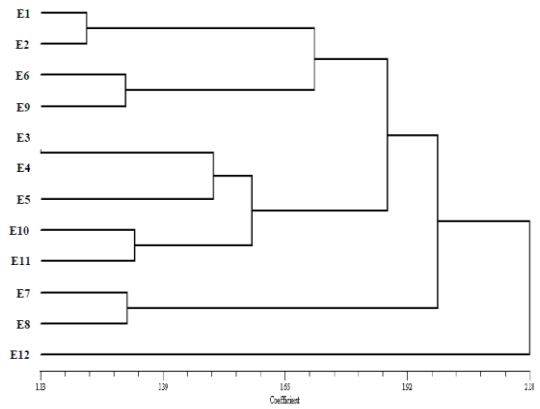

Fig. 3. Dendrogram of cluster analysis for *p. euphratica* ecotypes in Iran (E1=Jolfa, E2=Marand, E3=Maranjab, E4=Manjil, E5=Gonbad, E6=Sarakhs, E7=Hamidieh, E8=Dezful, E9=Mahalat, E10=Masumieh, E11=Gilvan, E12=Mahneshan).

Conclusions

The results of the banding pattern of DNA amplification with SSR primer pairs showed high genetic diversity inter-population in under-studied ecotypes. In other words, about 97% of the total molecular variance was covered by inter-population diversity. High variation of inter-population reflects the high value of sustainability in the ecotypes. There was Hardy-Weinberg's equilibrium in the ecotypes, which confirmed genetic stability in the ecotypes; the proximity of the values observed heterozygosis (Ho) and expected heterozygosis (He) in most ecotypes, confirmed the Hardy-Weinberg's equilibrium in them. Generally, the results of this study showed that genetic distance intra-populations were relatively low. Cluster analysis showed grouping of ecotypes did not follow any particular trend, and genetic distance was not correlated with geographical distance. Overall, the results of the present study indicated that *P. euphratica* stands covered vast areas of Iran in the past and probably had been contiguous; but today they have become separated due to various reasons. It seems vast areas in Iran are potentially ready for revival of *P. euphratica* stands. They can be pioneers and of major influence in the sustainability of ecosystems.

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