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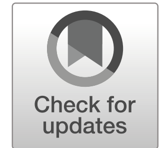

Consequences of habitat fragmentation on genetic diversity and structure of *Salix alba* L. populations in two major river systems of Turkey

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Abstract

Salix alba L. (white willow) is an indicator species of a healthy riparian ecosystem with great renewable energy potential in Turkey though habitats of the species in many river ecosystems are highly degraded or fragmented. Impacts of this degradation of river ecosystems on the magnitude and pattern of genetic diversity are not known. This study was aimed at assessing the genetic structure of *S. alba* populations in two highly degraded and fragmented river systems (the Göksu and Kızılırmak rivers) in Turkey with the use of 20 nuclear simple sequence repeat (SSR) loci. Fifteen of them were used for the first time in this study. Out of the 20 SSR loci, 10 loci significantly deviated from the Hardy-Weinberg equilibrium frequencies and five of them contributed greatly to the differentiation of populations. Generally, high levels of genetic diversity were found in populations of both Göksu and Kızılırmak river systems and moderate genetic differentiation ($F_{ST} = 0.07$) between the river systems. On the contrary to expectations, genetic diversity was higher in middle populations of the rivers (Ho = 0.67 of GRMID (Göksu river middle population), Ho = 0.68 in KRMID1 (Kızılırmak river middle population 1), and 0.65 in KRMID2 (Kızılırmak river middle population 2)) than in downstream populations (Ho = 0.65 in GRDOWN (Göksu river downstream population), Ho = 0.62 in KRDOWN1, 2 (Kızılırmak downstream populations 1, 2)). These could be due to experienced past bottlenecks, extensive vegetative material movements, and habitat fragmentation by constructed dams in the natural ecosystems of the two river systems. The genetic structure results revealed that the white willow populations in the two different river systems may have evolved from two different founder populations. A low level of genetic admixture between the river systems but high admixture within the river systems were observed due to extensive human-mediated vegetative material movements. The current study has provided valuable genetic data and information that could contribute insights to efficient conservation, management, utilization, and breeding of genetic resources of the species.

Keywords Genetic diversity · Simple sequence repeat (SSR) · Population structure · *Salix alba* · Habitat fragmentation

Introduction

Willow (*Salix* L.) species are distributed mainly in temperate and cold regions of the Northern Hemisphere (Argus 1997) with great morphological variations (Hardig et al. 2010). The diversity centers of the genus are considered to be in China (275 species) and in the Russian Federation (125 species) (Liu 1999). There are 100 species in North America and 65 species in Europe (Argus 1997). When considering the functions of willows in river plantations, water quality, erosion control, and biodiversity conservation, willows are accepted as pioneer species. The contribution and scope of global *Salix* plantation are increased to have sustainable forestry and to provide willow wood materials for industry and rural development (Ball et al. 2005). Among the willow species, *Salix alba* L. (white

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willow) appears to have an important place for the pulp industry, biomass, phytoremediation, and landscaping (Skvortsov 1999; Mleczek et al. 2010; Esbrí et al. 2018).

There are 27 species of willow with wide-ranging natural distributions in Turkey (Terzioğlu et al. 2014). White willow, ranging from shrubs to large trees (Velioğlu and Akgül 2016), is widely distributed in almost all river basins of Turkey (Davis 1965; Avcı 1999), but willow habitats in river systems have been highly fragmented or eroded due to the construction of dams for irrigation and electricity production. White willow is one of the most important species of Turkish river ecosystems due to its economic and ecological importance. In Turkey, willows are traditionally planted in rows along margins of moist meadows and fields and are regularly pruned to produce small-sized fuel-wood and raw material for non-wood products such as livestock feeds (Velioğlu and Akgül 2016). There is an increased interest in its use as an effective phytoremediation tool for cleaning rivers, ecosystem rehabilitation efforts, and short rotation plantation for energy in the world. However, the potential of the species is underestimated in Turkey. Furthermore, poplar and willow species, including white willow, are not subjected to Turkish forestry regulations. Thus, long-term programs for biomass plantations, conservation, and breeding of white willow genetic resources are lacking in the country.

Up to now, studies related to white willow in Turkey have been limited to the establishment of a clone bank and assessment of clones with respect to growth performance (Tunçtaner 1990). Genetic diversity is an important element in the dynamics of populations, because it is directly relevant to the evolutionary potential of the populations (Hughes et al. 2008). Although genetic diversity of trees is a key component in biodiversity and important in the resilience and adaptation of ecosystems to climate change, there is no study to date dealing with genetic structure of the fragmented population of white willow. With the current study, by using nuclear microsatellite loci, the magnitude of genetic diversity and its structure in fragmented white willow populations from two major river systems (Göksu and Kızılırmak) were assessed. These two rivers flow into different seas without common river basins. The natural ecosystems and habitats of willows in the river systems have either disappeared or been highly fragmented due to various environmental and industrial factors such as building of dams, hydroelectric power stations, sandbars, and industrial and urban wastes. The Göksu river (260 km in length) flows into the Mediterranean Sea and harbors 7 dams and/or hydroelectric power plants, while the Kızılırmak river, the longest river (1355 km long), flows to the Black Sea with 15 dams and/or hydroelectric power plants built on it. Since the overall habitat and distribution of native white willow populations have decreased, it is currently highlighted that conservation of natural willow populations and related ecosystems is very important (Vries De 2001). With the help of genetic information generated in the current study, new conservation strategies and breeding programs dealing with white willow will be effectively implemented in the future.

Materials and methods

Plant material

Two hundred and fifty-nine *S. alba* L. genotypes (trees) were sampled from nine populations located in the Göksu and Kızılırmak river systems. There were 112 genotypes representing four populations from the Göksu river while 147 genotypes were sampled from five populations in the Kızılırmak river system. The sampled populations were selected to represent the "upstream," "middle, "and "downstream" sections of the rivers. Since willows are usually clonally reproduced and dispersed, a minimum distance (200 m) among the genotypes within a population was taken into consideration to avoid sampling from the same cohort. Global positioning system (GPS) was used to obtain the location of sampled trees during field work (Table 1 and Fig. 1).

DNA extraction, quantification, and selection of simple sequence repeat markers

Freshly collected leaves from selected genotypes from two river systems were put in silica gels in the field until they were brought to the laboratory. Silica gel-dried leaves were crushed and powdered with liquid nitrogen in mortar by pestle to be used later in DNA extraction. The samples were stored in a deep freezer at − 80 °C until DNA isolation. Genomic DNA was extracted with a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1987). The NanoDrop spectrophotometer (NanoDrop 2000, Thermo Scientific, USA) was used to quantify the concentration of DNA. The template DNA concentration was diluted to 20 ng/μl before it was used.

Twenty well-amplified and polymorphic microsatellite loci (simple sequence repeat markers or SSR loci) were selected from literature (Supplementary Table 1). The SSR loci coded as Sare03, Sare04, Sare08, SB24, SB80, SB194, SB96, SB233, SB243, SB265, SB493, W293, W784, gSIMCT011, gSIMCT024, and gSIMCT052 were specific to willow species (Lian et al. 2001; Barker et al. 2003; Stamati et al. 2003; Lin et al. 2009; Lauron-Moreau et al. 2013) while the markers coded as WPMS18, PMGC2709, PMGC2889, and PMGC2163 were developed for poplar species (Web Site of International *Populus* Genome Consortium 2014). Fifteen of these SSR markers were tested and used in *S. alba* for the first time in the current study (Table 2). After optimization of reaction components and PCR cycles, forward primers of all the

Table 1 Detailed geographic and topographic information on studied *S. alba* populations

River system	Population/code	Pop no.	Sampled genotypes (*N*)	Location	Latitude range (N)	Longitude range (E)	Altitude range (m)
Göksu	Göksu upstream/GRUP1	1	31	Mut	36° 84′ 03″ 36° 27′ 48″	33° 17′ 75″ 33° 07′ 24″	246–284
	Ermenek upstream/GRUP2	2	23	Ermenek	36° 57′ 43″ 36° 34′ 25″	33° 47′ 27″ 33° 03′ 12″	333–342
	Mut midstream/GRMID	3	28	Mut	36° 56′ 99″ 36° 22′ 21″	33° 47′ 50″ 33° 25′ 19″	91–104
	Silifke downstream/GRDOWN	4	30	Silifke	36° 43′ 53″ 36° 25′ 38″	33° 76′ 42″ 33° 44′ 47″	27–58
Sub-total			112				
Kızılırmak	Kayseri upstream/KRUP	5	33	Kayseri/Ürgüp	38° 83′ 20″ 38° 71′ 19″	35° 22′ 52″ 34° 67′ 47″	789–1113
	Kırsehir midstream/KRMID1	6	32	Kırşehir	40° 08′ 78″ 39° 61′ 52″	33° 48′ 62″ 33° 43′ 52″	640–816
	Kırıkkale midstream/KRMID2	7	52	Kırıkkale	39° 69′ 17″ 38° 22′ 94″	34° 98′ 85″ 32° 97′ 79″	730–1269
	Corum downstream/KRDOWN1	8	15	Çorum	41° 09′ 71″ 41° 00′ 10″	35° 75′ 73″ 34° 41′ 80″	358–424
	Bafra downstream/KRDOWN2	9	15	Bafra	41° 69′ 01″ 41° 60′ 02″	35° 93′ 65″ 35° 90′ 44″	0–2
Sub-total			147				
Total			259				

loci were resynthesized fluorescently by the SACEM Company (Ankara) in order to discriminate polymerase chain reaction (PCR) products during the verification of fragment analysis.

For Sare03, Sare04, Sare08, SB80, gSIMCT011, gSIMCT052, and PMGC2163 SSR loci, the PCR reactions were performed with a 20-μl total volume containing 5 μl 5× HOTFIRE Pol Blend Master Mix (here on, it is referred to as Master Mix for short (Solis BioDyne, Tartu, Estonia)), 0.8 μl of each primer pair, 6 μl template DNA (20 ng/μl), and 7.4 μl double distilled water in 0.2-ml sterile Eppendorf tubes. For SB194,

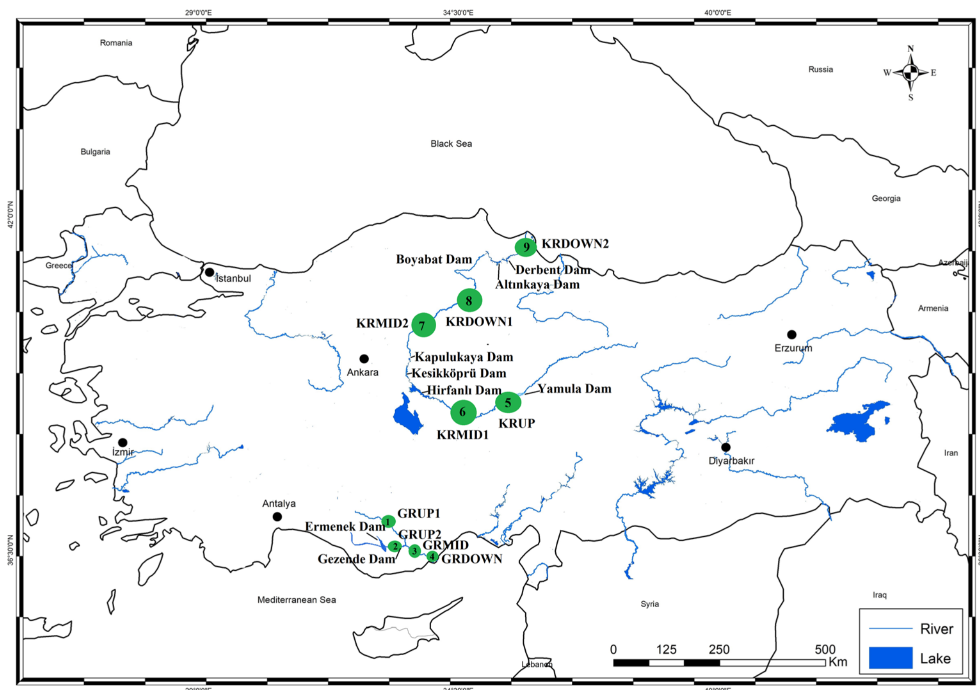

Fig. 1 Map showing the locations of studied populations. Please see Table 1 for the population codes (population numbers 1 through 4 and 5 through 9 represent Göksu and Kızılırmak river system, respectively)

Table 2 Descriptive statistics by loci

SSR locus	*N*	Na ± Se	Ne ± Se	Ar	PIC	Ho ± Se	He ± Se	F_{IS} ± Se
Sare03	28.78 ± 3.70	14.00 ± 0.62	7.83 ± 0.49	10.59	0.92	0.80 ± 0.04	0.87 ± 0.01	0.08 ± 0.05
Sare04	28.78 ± 3.70	11.78 ± 1.01	6.19 ± 0.47	9.92	0.90	0.78 ± 0.05	0.83 ± 0.01	0.06 ± 0.06
Sare08	28.78 ± 3.70	11.33 ± 0.96	6.51 ± 0.43	9.03	0.88	0.78 ± 0.06	0.84 ± 0.01	0.07 ± 0.07
SB24	28.78 ± 3.70	8.56 ± 0.47	4.65 ± 0.37	7.72	0.85	0.74 ± 0.08	0.77 ± 0.02	0.04 ± 0.10
SB80	23.33 ± 3.74	3.11 ± 0.26	1.74 ± 0.18	3.16	0.35	0.35 ± 0.08	0.37 ± 0.07	0.01 ± 0.09
SB194	28.78 ± 3.70	3.00 ± 0.00	2.23 ± 0.06	2.82	0.47	0.59 ± 0.05	0.55 ± 0.01	−0.08 ± 0.09
SB196	28.78 ± 3.70	1.67 ± 0.17	1.05 ± 0.02	1.37	0.05	0.05 ± 0.02	0.05 ± 0.02	−0.04 ± 0.01
SB233	28.67 ± 3.70	10.78 ± 0.97	3.79 ± 0.32	7.15	0.74	0.73 ± 0.03	0.72 ± 0.02	−0.02 ± 0.04
SB243	28.67 ± 3.70	4.56 ± 0.18	3.07 ± 0.12	3.90	0.64	0.86 ± 0.03	0.67 ± 0.01	−0.29 ± 0.05
SB265	28.78 ± 3.70	3.11 ± 0.26	1.47 ± 0.08	2.66	0.30	0.28 ± 0.04	0.30 ± 0.04	0.08 ± 0.05
SB493	28.78 ± 3.70	4.22 ± 0.46	2.38 ± 0.19	3.70	0.55	0.59 ± 0.06	0.55 ± 0.05	−0.08 ± 0.04
W293	28.67 ± 3.70	6.44 ± 0.58	2.88 ± 0.14	5.39	0.66	0.95 ± 0.02	0.65 ± 0.02	−0.48 ± 0.05
W784	28.78 ± 3.70	3.56 ± 0.24	2.30 ± 0.06	2.88	0.46	1.00 ± 0.00	0.56 ± 0.01	−0.78 ± 0.04
gSlMCT011	28.78 ± 3.70	3.89 ± 0.68	1.44 ± 0.11	3.19	0.28	0.21 ± 0.06	0.27 ± 0.06	0.23 ± 0.11
gSlMCT024	27.56 ± 3.59	4.67 ± 0.17	3.48 ± 0.20	4.39	0.71	0.60 ± 0.06	0.70 ± 0.02	0.15 ± 0.07
gSlMCT052	28.22 ± 3.42	13.67 ± 1.08	6.80 ± 0.56	9.57	0.88	0.75 ± 0.03	0.84 ± 0.02	0.10 ± 0.04
WPMS18	28.78 ± 3.70	4.00 ± 0.53	1.54 ± 0.14	3.30	0.34	0.23 ± 0.06	0.31 ± 0.06	0.32 ± 0.08
PMGC2709	28.78 ± 3.70	10.89 ± 0.9	4.97 ± 0.59	8.90	0.86	0.76 ± 0.03	0.77 ± 0.03	0.02 ± 0.03
PMGC2889	28.78 ± 3.70	9.89 ± 0.70	5.00 ± 0.30	7.45	0.81	0.87 ± 0.04	0.80 ± 0.01	−0.10 ± 0.05
PMGC2163	28.78 ± 3.70	6.56 ± 0.88	2.82 ± 0.19	5.45	0.63	0.88 ± 0.04	0.63 ± 0.02	−0.40 ± 0.06
Mean	28.40 ± 0.78	6.98 ± 0.32	3.61 ± 0.16		0.61	0.64 ± 0.02	0.60 ± 0.02	−0.06 ± 0.02

N = mean number of individuals with amplification, *Na* = mean number of different alleles, *Ne* = mean number of effective alleles, *Ar* = allelic richness, *PIC* = polymorphic information content, *Ho* = observed heterozygosity, *He* = expected heterozygosity, F_{IS} = inbreeding coefficient, *Se* = standard errors of estimates

SB96, SB233, SB265, W293, and W784 SSR loci, the reaction mixture contained 4 μl Master Mix, 0.5 μl each primer pair, and 10 μl water. On the other hand, 5 μl Master Mix, 0.5 μl of each primer pair, and 9 μl water were used for SB24, SB243, SB493, gSIMCT024, WPMS18, PMGC2709, and PMGC2889 SSR loci. Five-microliter template DNA (20 ng/μl) were added to PCR reactions involved in the last two groups of SSR loci.

PCR cycles were followed as 3 min at 94 °C for initial denaturation, then 30 cycles at 94 °C for 1 min of additional denaturation, at 50–55 °C (Ta) for 30 s of annealing, and at 72 °C for 30 s of extension and a final extension at 72 °C for 5 min for SB24, SB80, SB194, SB196, SB233, SB243, SB265, SB493, W293, and W784 loci. For Sare03, Sare04, Sare08, gSIMCT011, gSIMCT024, gSIMCT052, WPMS18, PMGC2709, PMGC2889, and PMGC2163 loci, cycling conditions were at 94 °C for 3 min, then 30 cycles at 94 °C for 30 s, at 52–58 °C (Ta) for 45 s, and at 72 °C for 1 min and a final extension period at 72 °C for 10 min (Eppendorf Mastercycler, Eppendorf, Canada).

Amplification products (5 μl) were loaded in 3% agarose gel prepared with 1× Tris-Borate-EDTA (TBE) buffer solution and run in 1× TBE (0.4 M Tris Boric Acid EDTA) buffer at ~ 120 mA for at least 30 min. The amplified products were visualized under UV light (Vilber Lourmat, France). Thermo Scientific GeneRuler Low Range DNA Ladder was used to determine the product size. Finally, the gels were photographed by Alpha Imager Gel Documentation System (Alpha Innotech, San Leandro, CA, USA).

Assay procedure for SSR fragment analysis was done by the BM Labosis Company (Çankaya, Ankara). Analyses of the samples were performed with the Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA), using an internal standard size as a marker (The GeneScan 400HD ROX dye). Allele sizes were checked and scored manually from electropherograms using the Peak Scanner Software 2.0 (Applied Biosystems Inc., Foster City, CA).

Analysis of data

The distinct and identical multilocus genotypes (MLGs) among all sampled genotypes were determined with the GenClone 2.0 software (Arnaud-Haond and Belkhir 2007). The MICRO-CHECKER software with Bonferroni-adjusted 95% confidence interval was used to detect the existence of

genotyping failure such as null alleles, stuttering, large allele dropout, and typographic errors caused by DNA degradation, low DNA concentrations, and primer site mutations. The frequencies of null alleles (r) according to the Brookfield (1996) method were also estimated. Probability test (Guo and Thompson 1992) for each locus in the populations was conducted to test the Hardy-Weinberg equilibrium (HWE) via GENEPOP software (Raymond and Rousset 1995; Rousset 2008). Linkage disequilibrium (LD) was estimated with the R *poppr* package (Kamvar et al. 2014) based on the index of association (Brown et al. 1980) proposed by Agapow and Burt (2001).

To be able to apply numerical analysis to latitude and longitude values, they were converted from "degrees/minutes/seconds (DMS)" units to "decimal degrees (DD)" units. Latitude, longitude, and altitude were evaluated as independent variables for He and Ho. Analysis of variance (ANOVA) was conducted to test if these topographical variables have a significant effect on estimated He and Ho.

To evaluate the amount of genetic differentiation among the nine populations, the population pairwise F_{ST} values (Slatkin 1995), their statistical significance, and the number of migrants (Nm) were estimated by using ARLEQUIN software (Excoffier and Lischer 2010) with the number of different allele distance method (number of permutations set to 1000).

The genetic structure of populations was determined by using the STRUCTURE software (Pritchard et al. 2000; Falush et al. 2003, 2007; Hubisz et al. 2009). Bayesian clustering methods were applied in two different assumptions: (i) one with a priori identification associated with population location and (ii) the other without a priori identification of specific grouping. Admixture ancestry and correlated allele frequency models (with $\lambda = 1$) were used in all runs. Run parameters consisted of 10 replicates each with 250,000 Markov Chain Monte Carlo (MCMC) replications after 50,000 burning length for $K = 1$ to 9 clusters. With the help of the STRUCTURE HARVESTER which is a web-based software, the ΔK statistics (true number of clusters) were calculated according to Evanno et al. (2005) and the graphic representation of these statistics was obtained by the CLUster Matching and Permutation Program (CLUMPP) software (Earl and vonHoldt 2012). The results from the CLUMPP were then used to find out the average membership coefficient matrices (Jakobsson and Rosenberg 2007). The output data of the CLUMPP was directly used as input data into the POPHELPER program (Francis 2017) to display graphical representation of population clusters. Lastly, a principal coordinate analysis (PCoA = classical multidimensional scaling, CMDS) was used to get further confirmation of the results of STRUCTURE analysis with the help of GenAlEx software (Peakall and Smouse 2012). The software helps to discover and visualize not only the similarities but also the dissimilarities of the data.

Analyses of molecular variance (AMOVA) were performed to partition the total genetic variation of white willow into components due to variation among river systems, among populations within a river system, and among genotypes within populations in a hierarchical form. To confirm the population subdivisions that were assumed by STRUCTURE, AMOVA was again carried out separately for Göksu and Kızılırmak rivers with the use of ARLEQUIN software (Excoffier and Lischer 2010).

Results

SSR loci and data assessment

Twenty SSR loci were found to be useful for detecting polymorphism in 259 white willow populations. Fifteen of 20 SSR loci were successfully amplified in white willow for the first time (Table 2). The percentage of missing data was found to be 1.3%. As reported by the results of MICRO-CHECKER software, there was no confirmation of mistyped allele sizes, typographic errors, and deviations from regular repeat motifs. Likewise, scoring errors due to large allele dropout were not observed in any locus across the nine populations.

Among the studied 20 SSR loci, it appeared that there were some loci with slightly high null allele frequencies for particular populations (Supplementary Table 2). Because of this, all analyses were carried out with and without null allele possessing loci in order to assess the genetic diversity and genetic differentiation of populations. Since there was no distinct change in estimated genetic diversity parameters, all loci were included in the analyses. Furthermore, after screening the studied genotypes with 20 loci, the results clearly indicated that there was no duplicated genotype existing in the sampled populations.

LD was assessed for the studied loci. The results revealed that all loci were in weak LD ($r_d = 0.0219$ at $P < 0.001$). The r_d value did not fall into the expected range from permutation tests (Fig. 2).

Genetic diversity of loci

When descriptive statistics by loci were examined, all loci were found to be polymorphic (Table 2). The means of observed and effective alleles were estimated as 6.98 and 3.61 alleles per locus, respectively. The SSR loci Sare03 and gSIMCT052 had the highest mean observed and effective numbers of alleles while the locus SB196 had the lowest values. Allelic richness (Ar) and polymorphic information content (PIC) of loci indicated that they varied in informativeness. Allelic richness varied from 1.37 in SB196 to 10.59 in Sare03 while PIC values ranged from 0.05 to 0.92 with a mean value of 0.61. There was only one locus (SB196) classified as low informative markers with the value of PIC < 0.25. Six of

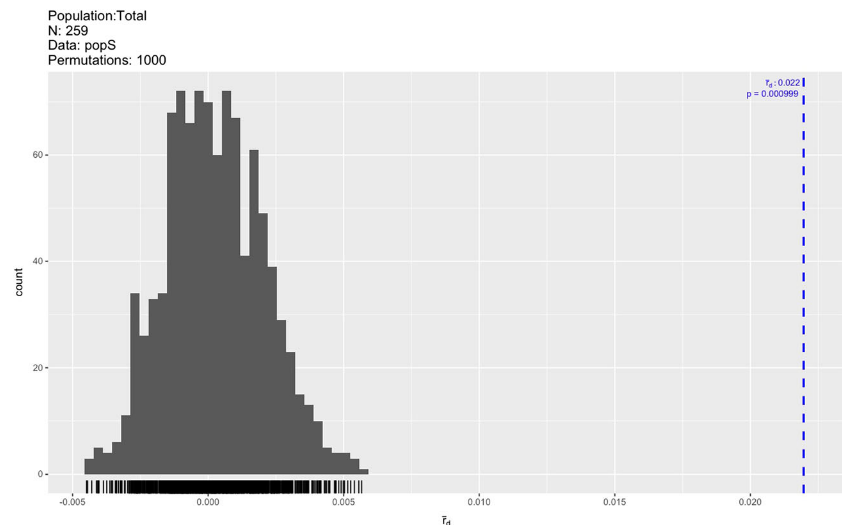

Fig. 2 Graph showing the estimated overall linkage disequilibrium. The r_d is the standardized index of association. Blue-dashed line indicates observed value

the 19 loci were evaluated to be moderately informative and the remaining loci to be highly informative (the PIC values exceeding 0.5). The changes in Ar and PIC values among different SSR loci follow the pattern as the heterozygosity level of loci. As a result, it was clear that the most diverse loci were found to be Sare03, Sare04, Sare08, and gSlMCT052 (Table 2).

The observed heterozygosity for the locus across populations varied from 0.05 in SB196 to 1.00 in W784 with an average of 0.64, while expected heterozygosity ranged from 0.05 in SB196 to 0.87 in Sare03 with an average of 0.60. The excess of heterozygosity was observed for 9 out of 20 loci, whereas the rest had positive F_{IS} values (Table 2). It was found that the loci Sare04, SB24, SB243, W293, W784, gSlMCT011, gSlMCT024, WPMS18, PMGC2889, and PMGC2163 showed significant deviations from the Hardy-Weinberg equilibrium. The highest F_{ST} values were estimated for the loci SB24, SB80, SB265, SB493, and PMGC2709, which contributed greatly to the differentiation of populations. The mean number of migrants (N_M) was found to be 5.00 with the highest contribution from locus W784 and the lowest from locus SB265 (Supplementary Table 3).

Genetic diversity of populations

Polymorphism in populations of the Göksu and Kızılırmak river systems was high (100% for Göksu and 96% for Kızılırmak). The mean observed number of alleles (Na) was 7.08 in the Göksu and 6.91 in the Kızılırmak river populations. The means of effective number of alleles (Ne) were 3.69 (ranged from 3.50 to 3.85) in the Göksu and 3.54 (ranged from 3.06 to 3.85) in the Kızılırmak river populations. The highest number of private alleles was found in the upstream population of the Göksu (GRUP1) and the middle population (KRMID2) of the Kızılırmak rivers.

In general, all studied populations had moderately high genetic diversity. The mean observed heterozygosity value of the Göksu river populations was 0.65 and varied from 0.61 in the upstream population (GRUP1) to 0.67 in the middle population (GRMID). Likewise, the mean observed heterozygosity value was 0.64 for the Kızılırmak river populations. It ranged from 0.62 (KRUP and KRDOWN1, KRDOWN2) to 0.68 (KRMID2). The mean of expected heterozygosity was moderate and ranged from 0.59 to 0.63 (average = 0.61) in the Göksu river populations and from 0.55 to 0.63 (average = 0.60) in the Kızılırmak river populations. All studied populations showed significant departure from Hardy-Weinberg equilibrium frequencies. The excess of heterozygosity was observed across all populations except for the downstream population of the Kızılırmak (KRDOWN2) and the upstream population of the Göksu river (GRUP1). The inbreeding coefficients (F_{IS}) for each population within river systems indicated that there was excess of heterozygotes ($F_{IS} = -0.05$ in the Göksu and -0.07 in the Kızılırmak river populations). Only one population from the Kızılırmak river system showed a slight presence of inbreeding ($F_{IS} = 0.03$). The genetic differentiation (F_{ST}) between two river systems was moderate (0.07). About 93% of genetic variation occurred within the river systems. Low to moderate levels of genetic differentiation existed among populations within the river systems ($F_{ST} = 0.02$ among the populations of Göksu river and $F_{ST} = 0.05$ among the Kızılırmak river populations) (Table 3).

The Garza-Williamson indices indicate whether a population experienced a past genetic bottleneck or not (Garza and Williamson 2001). All estimated Garza-Williamson index values for each of the nine populations were lower than < 0.68 which is the critical value. It was clear that there was a past reduction in effective population sizes of the species in both river systems (Table 3).

Effects of geographic factors such as longitude, latitude, and altitude on genetic diversity of white willow populations

Table 3 Estimated genetic diversity parameters for studied populations in the Göksu and the Kızılırmak river systems

	N	Na ± Se	Ne ± Se	Pa	P (%)	G-W index (M)	Ho ± Se	He ± Se	F_{is} ± Se	F_{st}	
Göksu river/populations											
GKSUP1	31	7.75 ± 1.12	3.85 ± 0.58	15	100.00	0.32	0.61 ± 0.07	0.59 ± 0.06	0.00 ± 0.07*	0.02	0.07
GKSUP2	23	6.40 ± 0.81	3.50 ± 0.45	8	100.00	0.31	0.66 ± 0.07	0.60 ± 0.05	− 0.09 ± 0.07*		
GKMID	28	7.00 ± 0.94	3.67 ± 0.43	6	100.00	0.34	0.67 ± 0.06	0.63 ± 0.05	− 0.04 ± 0.07*		
GKSDOWN	30	7.15 ± 0.97	3.74 ± 0.55	9	100.00	0.32	0.65 ± 0.07	0.60 ± 0.06	− 0.08 ± 0.07*		
Mean	28	7.08 ± 0.96	3.69 ± 0.50	–	100.00	0.32	0.65 ± 0.07	0.61 ± 0.06	− 0.05 ± 0.07*		
Kızılırmak river/populations											
KRUP	33	7.85 ± 1.10	3.88 ± 0.55	11	95.00	0.33	0.62 ± 0.06	0.61 ± 0.06	− 0.03 ± 0.07*	0.05	
KRMID1	32	7.50 ± 1.07	3.85 ± 0.55	9	100.00	0.31	0.68 ± 0.06	0.63 ± 0.05	− 0.08 ± 0.06*		
KRMID2	52	8.25 ± 1.09	3.32 ± 0.43	19	95.00	0.30	0.65 ± 0.07	0.58 ± 0.06	− 0.12 ± 0.06*		
KRDOWN1	15	5.35 ± 0.77	3.06 ± 0.41	1	90.00	0.34	0.62 ± 0.07	0.55 ± 0.06	− 0.13 ± 0.08*		
KRDOWN2	15	5.60 ± 0.60	3.59 ± 0.42	6	100.00	0.32	0.62 ± 0.07	0.63 ± 0.05	0.03 ± 0.08*		
Mean	29.40	6.91 ± 0.93	3.54 ± 0.47	–	96.00	0.32	0.64 ± 0.07	0.60 ± 0.06	− 0.07 ± 0.07*		
Total	28.70	6.98 ± 0.32	3.61 ± 0.16		97.78 ± 1.21%	0.32	0.64 ± 0.02	0.60 ± 0.02	− 0.06 ± 0.02		

N = number of individuals, Na = mean number of different alleles, Ne = mean number of effective alleles, Pa = private alleles, $\%P$ = percentage of polymorphic loci, *G-W* index (M) = Garza-Williamson index, Ho = observed heterozygosity, He = expected heterozygosity, F_{st} = fixation index, F_{st}= the inbreeding coefficient within subpopulations, Se = standard errors of estimates

*$P < 0.05$

were represented in Fig. 3. Analysis of variance with these variables indicated that these geographic factors did not have significant effects on estimated He or Ho of the studied populations in the Kızılırmak river system ($P > 0.05$).

Genetic differentiation and structuring of populations

Pairwise genetic distance values (F_{ST}) among populations varied from 0.010 (between middle populations (KRMID1, KRMID2 of the Kızılırmak river)) to 0.11 (between downstream populations (KRDOWN1, KRDOWN2 of the Kızılırmak river)). The downstream populations of the Kızılırmak river (KRDOWN1, KRDOWN2) were genetically most distant to other populations. The highest number of migrants (N_M) was found between the upstream (GRUP1) and downstream populations (GRDOWN) of the Göksu river as 20.62 and between middle populations (KRMID1, KRMID2) of Kızılırmak as 24.38 (Supplementary Table 4).

There were no differences in the estimation of delta K values between the two methods (with and without prior information on the geographic distribution of genotypes) when data from river systems were combined. The result of the analysis pointed out the existence of two main clusters based on the delta K value of nine populations from the Göksu and the Kızılırmak rivers. The graphical representation of delta K with the Evanno method is shown in Supplementary Fig. 1. The first cluster consisted of members of the Göksu river populations (97.47% of genotypes) except for three genotypes which ended up in the Kızılırmak river while all genotypes of the Kızılırmak river were placed into the second cluster with a 100% membership value. It was

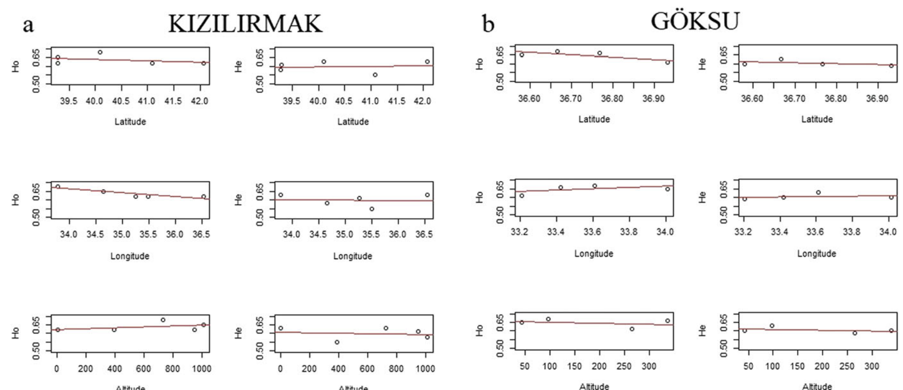

Fig. 3 The scatter plots and best fit lines (linear regression lines) represent Ho (observed heterozygosity) and He (expected heterozygosity) vs. latitude, longitude, and altitude of the studied population in the Göksu (**a**) and Kızılırmak rivers (**b**)

clear that there was a significant spatial effect on the genetic structuring of populations from two river systems (Fig. 4).

When STRUCTURE analysis for only the Göksu river was carried out, 112 genotypes from four different populations were placed into two major clusters (Supplementary Fig. 2). Fifty-seven of the 112 sampled genotypes were in the first cluster and 55 in the second cluster. Two clusters did not correlate with the geographic origins of the genotypes with regard to the proportion of membership (Fig. 5). When the results of STRUCTURE analysis within the Kızılırmak river system were taken into consideration, the maximum delta K was detected as 2 (Supplementary Fig. 2). Thirty-nine of 147 sampled genotypes formed the first cluster, whereas 108 genotypes were in the second cluster. All genotypes from the downstream populations were placed in the second cluster (Fig. 5).

The results of PCoA with nine populations revealed that 74% of the total variation was explained by the first three axes with 30%, 23%, and 21%, respectively. The Göksu and Kızılırmak river populations were separated by the first principal coordinate (30%). However, one downstream population (KRDOWN1) was an exception in that it was separated by the first (30%) and second axes (23%). This downstream population (KRDOWN1) seemed to be the most distant to the other populations (Fig. 6). The genetic grouping of populations from both river systems followed the same results from the PCoA when the analyses were repeated by STRUCTURE and pairwise F_{ST} based on neighbor joining method.

Partition of total genetic diversity

The results of global locus-by-locus (no. of different alleles, F_{ST}) analyses of molecular variance (AMOVA) revealed that there was a significant differentiation ($P < 0.001$) among nine populations from the two river systems. The fixation index values ($F_{ST} = 0.07$) were significant and consistent with the pairwise F_{ST} results. Small portion of the total variation was found to be among river systems (3.27%) and among the population within the river system (3.47%). The great portion of the total variation was among genotypes within the population in the two rivers (93.26%) (Table 4).

The Göksu river populations were subdivided into two major groups depending on both the distance method and the Bayesian analysis. The Ermenek population (GRUP2), tributary of the Göksu river, formed one group while the upstream (GRUP1), middle (GRMID), and downstream (GRDOWN) populations formed another group. When AMOVA was carried out later, the total variance that accounted for river sections was found to be insignificant (0.86%). A great portion of the total variance was found to be among genotypes within the populations of river sections. Based on both the distance method and the Bayesian analysis, two major groups were also detected in the Kızılırmak river system. One group was composed of the upstream (KRUP) and middle populations (KRMID1 and KRMID2). The other group included the downstream populations (KRDOWN1 and KRDOWN2). AMOVA results showed that 93.94% of the total variation was attributed to genotypes within the population. Only 3.44% and 2.82% of the total variation were due to populations within river sections and among river sections, respectively (Table 4).

Discussion

This is the first study dealing with the magnitude and structuring of genetic diversity of white willow populations in two ecologically different river systems exposed to intensive human activities for so long. The study revealed important findings on the structuring of genetic diversity of populations and ecological and evolutionary factors that affect the genetic composition of the species in connection with the establishment of future conservation and breeding strategies.

Studied SSR loci developed for different *Salix* species appeared to be polymorphic and useful for genetic characterization of genetic resources of white willow. Allele size ranges of

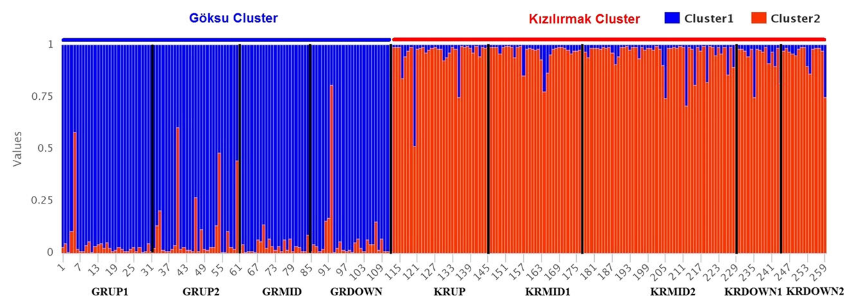

Fig. 4 Graphical representation of the cluster membership of 259 *S. alba* genotypes from nine populations sampled from the Göksu and Kızılırmak rivers. The graph indicates two inferred clusters and their associations with populations. The different clusters were represented with different colors. Vertical lines separate the populations within the river systems. Please see Table 1 for the codes of the populations

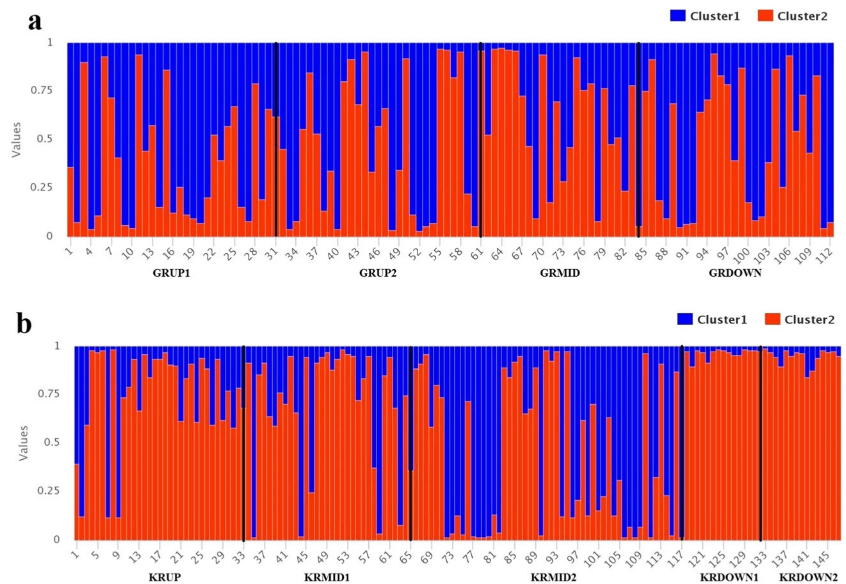

Fig. 5 **a** Graphical representation of the cluster membership of 112 *S. alba* genotypes sampled from the Göksu river. **b** Graphical representation of the cluster membership of 147 *S. alba* genotypes sampled from the Kızılırmak river. Please see Table 1 for the codes of the populations

SSR loci identified in the current study are compatible with those reported in the previous studies (King et al. 2010; Singh et al. 2014; Rungis et al. 2017). Some loci indicated that there may be presence of null alleles, which were specific to certain populations. However, inclusion or exclusion of these loci did not affect the estimation of genetic diversity parameters. PCR failure caused by inconsistent DNA template quality or low template quantity is the potential causes of null alleles in this study. No clonal duplication among sampled genotypes was detected in the two river systems. This finding is not surprising since similar results were reported in other *Salix* species (Stamati et al. 2007; Steltzer et al. 2008; Douhovnikof et al. 2010; Kikuchi et al. 2011; Sitzia et al. 2018). Even though *Salix* species are reproduced easily with vegetative materials and transferred downstream of rivers (Rood et al. 2003; Kuzovkina et al. 2008), they rarely produce root suckers. Thus, it was obvious from the results of the current study that white willow is dispersed mainly by sexual reproduction in both the Göksu and Kızılırmak river systems.

In the current study, 16 of the 20 SSR loci exhibited high allelic richness (> 0.3). Only locus SB196 was found to be less

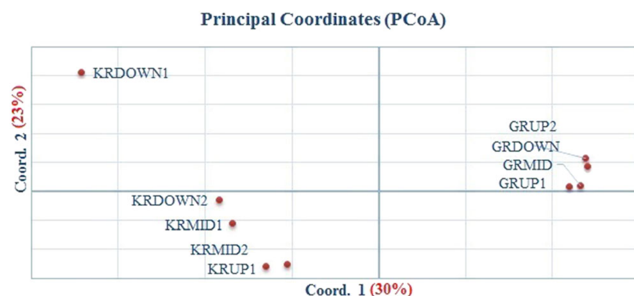

Fig. 6 Principal coordinate analysis (PCoA) with nine populations based on Nei's (1972) genetic distances showing differentiation of populations. Please see Table 1 for the codes of the populations

informative (< 0.25). Thirteen of 20 SSR loci were informative with high PIC values (> 0.5). We recommend that the Sare03, Sare04, Sare08, SB24, SB233, SB243, W293, gSIMCT024, gSIMCT052, PMGC2709, PMGC2889, and PMGC2163 loci can be used effectively in the future genetic studies dealing with white willow or other related willow species. Although SB194, SB243, and SB80 loci had higher allelic richness parameters (Ne, PIC, and He), previous studies (King et al. 2010; Singh et al. 2013a, b, 2014; Rungis et al. 2017) did not report similar results due to differences in the number of SSR loci, sample sizes of populations, and their geographic locations. Also, geographically distant populations which are exposed to different environmental conditions and human-related activities may have contributed to a different evolutionary history in white willow. The excess of heterozygosity was observed in 9 out of 20 loci. Five of these deviated significantly from the Hardy-Weinberg equilibrium. Excess of heterozygosity may be due to selection and dispersal of trees with heterozygote superiority by human-assisted migration. There were also five loci with excess of homozygosity, significantly deviating from the Hardy-Weinberg equilibrium due to inbreeding and the population sub-structuring caused by loss and fragmentation of natural habitats. The highest contributions to mean F_{ST} values, in turn, differentiation of populations, were found to be from the loci SB24, SB80, SB265, SB493, and PMGC2709 with low number of migrants (N_M). To reduce the cost of similar studies and to increase efficiency of studies in the future, these loci could be preferred to study genetic characterization of white willow populations.

All studied populations were found to be highly polymorphic with the presence of a high level of allelic diversity. The highest number of private alleles was found in the upstream population of the Göksu river (GRUP1, 15 private alleles) and

Table 4 Analyses of molecular variance (AMOVA) based on SSR loci *S. alba*

	Sum of squares	Variance components	Percentage of total variation	Fixation indices
Source of variation				
Among rivers	74.69	0.22	3.27	$F_{CT} = 0.03$*
Among populations within river	129.35	0.23	3.47	$F_{SC} = 0.04$*
Within populations	3093.18	6.14	93.26	$F_{ST} = 0.07$*
Total	3297.22	6.58	100	–
Source of variation/Göksu river				
Among river sections	15.21	0.05	0.86	$F_{CT} = 0.008$*
Among populations within river	24.56	0.10	1.64	$F_{SC} = 0.02$*
Within populations	1353.19	6.19	97.50	$F_{ST} = 0.02$
Total	1392.96	6.34	100	–
Source of variation/Kızılırmak river				
Among river sections	31.71	0.18	2.82	$F_{CT} = 0.03$*
Among populations within river	57.87	0.22	3.44	$F_{SC} = 0.04$*
Within populations	1739.99	6.08	93.94	$F_{ST} = 0.06$*
Total	1829.57	6.49	100	–

F_{CT} = differences among river systems, F_{SC} = difference among populations within rivers, F_{ST} = differences within populations, F_{CT} = differences among river sections, F_{SC} = difference among populations within river sections, F_{ST} = differences within populations

*$P < 0.05$

in the middle population (KRMID2, 19) of the Kızılırmak river. Apparently, these populations maintained original genetic diversity through harboring larger intact habitats. They may be considered as the major genetic diversity areas along two river systems for future genetic resource conservation or selection practices related to tree improvement.

In general, high levels of genetic diversity were found in populations of both the Göksu and Kızılırmak rivers. Mean observed heterozygosity (Ho = 0.64) was found to be higher than mean expected heterozygosity (0.60). This was also true for almost all studied populations of the river systems except for the downstream population (KRDOWN2) in the Kızılırmak river. Previous studies conducted with *Salix* species also reported similar results, that is, excess of heterozygosity (*Salix eriocephala*, Lauron-Moreau et al. 2013; *Salix caprea*, Perdereau et al. 2014; *Salix hukaoana*, Kikuchi et al. 2011; *Salix daphnoides*, Sochor et al. 2013). This high heterozygosity could be due to negative assortative mating, selection (heterozygote advantage), or the proportion of heterozygote genotypes in founder populations. Excess of heterozygosity was also evident from estimated Garza-Williamson indices for populations (less than critical value of 0.68). Low values suggested that a reduction in population size occurred due to an experienced genetic bottleneck in the past coupled with random genetic drift eliminating low frequency alleles. Allelic diversity is generally lost faster than heterozygosity when a population undergoes bottleneck (Nei et al. 1975). Furthermore, wind pollination, seed dispersal via animals, and vegetative material movements by locals could also contribute to increased heterozygosity in studied white willow populations.

In unfragmented natural river ecosystems, higher genetic diversity could be expected in mid-streams as well as downstream populations compared to upstream populations if seed and vegetative propagules are freely dispersed in river systems. This pattern seemed to be true for only middle populations of both river systems. Not observing a similar pattern in downstream populations may be explained by the lack of suitable habitats or interruptions of the natural river ecosystems by hydroelectric power stations and irrigation dams. Although downstream accumulation of genetic diversity in some species has been reported in different river systems (Lundqvist and Andersson 2001; Liu et al. 2006), the current study and studies with other *Salix* species in the literature have not supported these findings except for the study carried out by Kikuchi et al. (2011) on *S. hukaoana*. Discrepancies between the results of previous and current studies may be due to variation in effectiveness of seed and clonal material dispersal, effective population size, and degree of fragmentation in river ecosystems.

The genetic differentiation pattern of the populations within and between two river systems was found to be similar in all three analyses (STRUCTURE, AMOVA, and PCoA). Two hundred and fifty-nine white willow genotypes were placed to two genetic clusters by genetic STRUCTURE analysis. It appeared clearly that white willow populations in two different river basins originated from two different founder populations. Genetic

differences are often correlated with geographic distance between populations, so it seems that geographical distance has an important effect on genetic differentiation of white willow populations in the current study. Geographical barriers such as mountains (Taurus Mountain ranges) are likely to contribute to the differentiation of populations of two river systems by preventing extensive gene flow ($F_{ST} = 0.07$). Although genetic differentiation between white willow populations of Göksu and Kızılırmak river systems was found to be moderate ($F_{ST} = 0.07$), a low level of genetic admixture was still present in reproductively isolated populations of two rivers. Similar previous studies dealing with different willow species have reported the genetic differentiation of populations between river systems as low or moderate (Puschenreiter et al. 2010; Trybush et al. 2012; Sochor et al. 2013; Berlin et al. 2014; Perdereau et al. 2014; Ukwubile et al. 2014). All members of the family Salicaceae generally display a low F_{ST} value among the populations when compared with other forest trees as a result of small, light seeds readily dispersed by wind and water and vegetative propagation (Ciftci et al. 2017). Although long-distance gene flow is not common in willow species due to short viability of the seed, human-mediated vegetative material movements lead to genetic similarities of populations at distant locations. The Göksu river basin is located on one of the major migratory routes of people moving from the Mediterranean region to central Turkey (where the Kızılırmak river cuts through its large part) or from central Turkey to the Mediterranean regions. The long history of human migration is likely to contribute genetic material exchange between the two river systems so that a low level of admixture between river systems is still present.

When genetic structures of the Göksu and Kızılırmak river systems were examined separately, the populations of both rivers seemed to have two genetic clusters with a high level of admixtures among populations, especially in the Göksu river. Altitude differences and the Gezende Dam may cause slight differentiation of the Ermenek population from other populations in the Göksu river. Despite the presence of geographic barriers, four populations of the Göksu river were genetically close to each other due to the high level of gene flow among subpopulations. Even though willow seeds stay viable only for a few weeks and vulnerable to long-distance dispersal (Maroder et al. 2000), seed dispersal via hydrochory (dispersion by water) and anemochory (dispersion by wind) (Imbert and Lefèvre 2003) or a combination of both and human-assisted vegetative material dispersals may have played an important role in long-distance gene flow among white willow populations in the Göksu river system.

In the Kızılırmak river system, upstream (KRUP) and middle populations (KRMID1 and KRMID2) indicated higher admixture while the downstream populations (KRDOWN1 and KRDOWN2) had a homogeneous structure. The reason for finding high admixture in the midstream populations (KRMID1 and KRMID2) could be explained by human-assisted material dispersal. These middle populations of the river are located in the Kızılırmak river section where human population and demand for fuel-wood and building material are high. Interestingly, the KRDOWN1 and KRDOWN2 populations were found to be highly diverged within the Kızılırmak river system, as well as genetically the most distant ones to the populations of the two river systems. Considering the locations of these downstream populations where there are less human impacts and habitat fragmentations, it is comprehended that the genetic structure of the populations may not be affected extensively by human-assisted material dispersal. Additionally, several constructed dams (Boyabat, Altınkaya, and Derbent Dams) between the KRDOWN1 and KRDOWN2 populations interrupting the natural river flow may have also contributed to their genetic differentiation.

Conclusion

- Fifteen of 20 SSR loci were used for the first time in the current study and 13 of them were highly informative. We recommend that these SSR loci could be effectively used in future genetic diversity and characterization studies in white willow.
- In general, high levels of genetic diversity were found in populations of both the Göksu and Kızılırmak river systems; genetic diversity was especially high in middle populations of the rivers. There was an excess of heterozygosity in populations between and within river systems. These findings could be due to experienced past bottlenecks, extensive vegetative material movements, and habitat fragmentation in natural ecosystems of the rivers.
- Although genetic differentiation between white willow populations of the Göksu and Kızılırmak river systems was found to be moderate ($F_{ST} = 0.07$), a low level of genetic admixture was found in reproductively and geographically isolated populations of the two rivers. The presence of the high level of genetic admixture within the river systems is most likely to be caused by human-mediated vegetative material movements.
- The results of the study demonstrate that white willow populations in two different river systems may have evolved from two different founder populations.
- This study provides important insights for efficient conservation, management, utilization, and breeding of

genetic resources of economically valuable white willow. In order to conserve existing riparian ecosystems of willow species, effective legislation on the conservation issue should be established and a proper management policy should be put into action for setting up in situ conservation areas.

Data archiving statement The data used in the study have been formatted according to the requirements of Species Database of Tree Genes (http://dendrome.ucdavis.edu/treegenes/). The data will be deposited in Tree Genes on acceptance.

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