DIFFERENTIAL IMPACTS OF HABITAT FRAGMENTATION ON GENETIC
STRUCTURE OF TWO IMPORTANT RIPARIAN SPECIES (*SALIX ALBA &
POPULUS NIGRA*)

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DIFFERENTIAL IMPACTS OF HABITAT FRAGMENTATION ON GENETIC STRUCTURE OF TWO IMPORTANT RIPARIAN SPECIES (*SALIX ALBA & POPULUS NIGRA*)

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ABSTRACT

**DIFFERENTIAL IMPACTS OF HABITAT FRAGMENTATION ON
GENETIC STRUCTURE OF TWO IMPORTANT RIPARIAN SPECIES
(*SALIX ALBA & POPULUS NIGRA*)**

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Salix alba and *Populus nigra* are two important tree species of riparian ecosystems. With a great ecologic and economic significance, they are naturally distributed in almost all river basins of Turkey. The genetic structures of these species in two river basins in Turkey were studied using eight common microsatellite markers to both species to reveal the impacts of habitat fragmentation. Therefore, the studied rivers were selected to represent a highly fragmented river, and a protected river from habitat fragmentation which are respectively Kızılırmak River and Melendiz River of Ihlara Valley. For the study, 132 *S. alba* and 84 *P. nigra* genotypes were sampled from four different populations of Kızılırmak while 29 *S. alba* and 25 *P. nigra* genotypes were sampled from Ihlara.

Various population genetics analyses were performed with the obtained data to evaluate the efficiency and diversity of microsatellite loci, genetic diversity and structure of the populations of two species, effects of habitat fragmentation, and other possible human-related activities on these species. The results of these analyses demonstrated that habitat fragmentation and other human-mediated activities had important effects on the genetic structures and diversities of both species. Differentially, *P. nigra* was detected as it was affected by these human activities

more than *S. alba*. The reason behind this is that *P. nigra* has been more commonly transported by humans and commercially cultivated in Anatolia by comparing to *S. alba*. The results indicate that the expected diversity levels were low (mean uHe values are 0.61 for *S. alba* and 0.59 for *P. nigra*) and the excess of observed heterozygosity levels was found (mean Ho values are 0.63 for *S. alba* and 0.87 for *P. nigra*). This suggests the recent bottleneck events causing the loss of allelic diversity in both species. The difference between Ho and uHe values was greater for *P. nigra* while these values were closer to each other for *S. alba*. It seems the populations of *S. alba* were close to the Hardy-Weinberg equilibrium which could be caused by less severity of past experienced bottleneck events of the species. Furthermore, the differentiation between Kızılırmak and Ihlara populations was found to be highly significant for both species (F_{ST} values are 0.05 and 0.14 for *S. alba* and *P. nigra*, respectively). Four populations of Kızılırmak were also differentiated from each other significantly for both species (F_{ST} values are 0.02 for both *S. alba* and *P. nigra*). This difference between the F_{ST} values also points out the wild nature of *S. alba* populations is still maintained, but the high vulnerability of *P. nigra* populations to habitat fragmentation and other human-related activities.

Genetic resources of these two important species of riparian ecosystems need to be protected from unregulated human activities including habitat destruction and fragmentation caused by several agricultural and urbanization practices, and improper afforestation and cultivation policies. The information generated from this study is valuable for developing both successful conservation and breeding programs, and new ways to decrease the negative impacts of habitat fragmentation on these two species.

Keywords: *Salix alba, Populus nigra*, Habitat Fragmentation, Genetic Diversity, Genetic Structure

ÖZ

HABİTAT FRAGMENTASYONUNUN NEHİR EKOSİSTEMİNİN İKİ ÖNEMLİ AĞAÇ TÜRÜNÜN (*SALİX ALBA & POPULUS NİGRA*) GENETİK YAPILARI ÜZERİNE FARKLI ETKİLERİ

Durgut, Bilge
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Salix alba ve *Populus nigra*, büyük ekolojik ve ekonomik öneme sahip, nehir ekosistemlerinin iki ağaç türü olup, Türkiye'nin hemen hemen tüm nehir havzalarında doğal olarak yayılış göstermektedir. Habitat fragmentasyonunun etkilerini ortaya çıkarmak için Türkiye'deki iki nehir havzasından alınan örneklerle bu türlerin genetik yapıları, iki tür için ortak olan sekiz mikrosatelit belirteci kullanılarak incelenmiştir. İncelenen nehirler, oldukça parçalanmış ve habitat parçalanmasından korunan nehirleri temsil edecek şekilde sırasıyla Kızılırmak Nehri ve Ihlara vadisindeki Melendiz Nehri olarak seçilmiştir. Çalışma için Kızılırmak'ın dört farklı popülasyonundan 132 *S. alba* ve 84 *P. nigra* genotipi, Ihlara'dan ise 29 *S. alba* ve 25 *P. nigra* genotipi örneklenmiştir.

Elde edilen verilerle mikrosatelit lokuslarının etkinliğini ve çeşitliliğini; iki türün popülasyonlarının genetik çeşitliliğini ve yapısını; habitat fragmentasyonunun ve diğer olası insan faaliyetlerinin bu türler üzerindeki etkilerini değerlendirmek için çeşitli analizler yapılmıştır. Bu analizlerin sonuçları, habitat fragmentasyonunun ve diğer insan kaynaklı faaliyetlerin her iki tür için de genetik yapıları ve çeşitlilikleri olumsuz yönde değiştirdiğini göstermiştir. Farklı olarak, *P. nigra*'nın bu insan

aktivitelerinden *S. alba*'dan daha fazla etkilendiği tespit edilmiştir. Bunun nedeni, *P. nigra*'nın Anadolu'da *S. alba*'ya göre daha yaygın olarak insanlar tarafından taşınmış ve ticari olarak yetiştirilmiş olmasıdır. Analiz sonuçları, düşük beklenen genetik çeşitlilik (ortalama uHe değerleri *S. alba* için 0,61 ve *P. nigra* için 0,59) ve gözlemlenen heterozigotluk fazlalığı (ortalama Ho değerleri *S. alba* için 0,63 ve *P. nigra* için 0,87) olduğunu göstermektedir. Bu, her iki türün de heterozigotlukta fazlalık ve alelik çeşitlilikte kayıplara neden olan yakın darboğaz olayları yaşadıklarını ortaya çıkarmıştır. Ho ve uHe değerleri arasındaki farklar *P. nigra*' da *S. alba*'ya göre daha fazladır. Bu durum, geçmişte yaşanan darboğaz olaylarının *S. alba* popülasyonları üzerindeki etkilerinin daha az olmasından ötürü *S. alba* popülasyonlarının Hardy-Weinberg dengesine yakın olduğunu göstermektedir. Ayrıca, Kızılırmak ve Ihlara popülasyonlarının birbirinden önemli ölçüde farklılaştığı keşfedilmiştir (F_{ST} değerleri *S. alba* ve *P. nigra* için sırasıyla 0.05 ve 0.14'tür). Kızılırmak'ın dört popülasyonu kendi içlerinde karşılaştırıldığında da anlamlı değerler ile farklılaştıkları görülmüştür (F_{ST} değerleri hem *S. alba* hem de *P. nigra* için 0.02'dir). Bu değerler arasındaki farklar da *S. alba*'nın yabani karakterlerinin daha iyi korunduğuna ve *P. nigra*'nın habitat fragmentasyonu ve diğer insan faaliyetlerinden daha fazla etkilendiğine işaret etmektedir.

Bu iki önemli türün gen kaynaklarının, çeşitli tarım ve kentleşme uygulamaları için habitat tahribatı ve fragmentasyonu, yanlış ağaçlandırma ve yetiştirme politikaları gibi bilinçsiz insan faaliyetlerinden korunması gerekmektedir. Bu çalışmadan elde edilen bilgiler, hem başarılı koruma ve ıslah programları geliştirmek hem de habitat parçalanmasının bu iki tür üzerindeki olumsuz etkilerini azaltmak üzerine yeni programlar geliştirmek için değerlidir.

Anahtar Kelimeler: *Salix alba, Populus nigra*, Habitat Fragmentasyonu, Genetik Çeşitlilik, Genetik Yapılanma

To my family and all beloved ones

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

ABBREVIATIONS

%P	Percentage of Polymorphic Loci
AMOVA	Analysis of Molecular Variance
Ar	Allelic Richness
bp	Base pairs
CLUMPP	Cluster Matching and Permutation Program
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraaceticacid disodium salt
F	Inbreeding Coefficients
F_{CT}	Difference Among Groups for the Total Population
F_{IS}	Inbreeding Coefficient Within Individuals
F_{IT}	Inbreeding Coefficient Within Total Population
F_{SC}	Differences Among Population Within Groups
F_{ST}	Inbreeding Coefficient Within Populations
GDA	Genetic Data Analysis
G-W Index (M)	Garza-Williamson Index
uHe	Unbiased Expected Heterozygosity
Ho	Observed Heterozygosity
HWE	Hardy–Weinberg Equilibrium
I	Shannon's Information Index
IHR-NEV	Ihlara population from Melendiz
IUCN	The International Union for Conservation of Nature
KIZDOWN-COR	Downstream Population of Kızılırmak-Çorum
KIZMID-KIR	Middle Population of Kızılırmak-Kırşehir
KIZMID-KRK	Middle Population of Kızılırmak-Kırıkkale
KIZUP-KAY	Upstream Population of Kızılırmak-Kayseri

MCMC	Markov Chain Monte Carlo
MgCl2	Magnesium Chloride
Na	Number of Alleles
Ne	Number of Effective Alleles
Nm	Number of Migrants
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PI	Probability of Identity
PIC	Polymorphism Information Content
R_{ST}	Inbreeding Coefficient Within Populations (based on size)
SSR	Simple Sequence Repeats
STR	Short Tandem Repeats
Ta	Annealing Temperature
TE	Tris EDTA
TUBIVES	Türkiye Bitkileri Veri Servisi
TÜBİTAK	The Scientific and Technological Research Council of Turkey
UPGMA	Unweighted Pair-Group Method with Arithmetic Mean

CHAPTER 1

INTRODUCTION

1.1   The Salicaceae Family

The Salicaceae family is a flowering plant family consisting of dioecious to hermaphrodite trees and shrubs with their approximately 480 species (Argus, 2010; Eckenwalder, 2010; Simpson, 2010). Members of Salicaceae are dominant species of riparian ecosystems throughout river basins of the northern hemisphere (Politti et al., 2018). The family has great importance on many ecosystems and also they are used as model organisms for several research areas such as molecular biology and genetics, plant domestication, and conservation (Kuzovkina & Vietto, 2014).

Salix (willow) and *Populus* (poplar) are traditionally considered as two constituent genera of the Salicaceae family (Eckenwalder, 1996). Recent studies show that these two genera are sister to each other constructing a truly natural monophyletic group that originates from a common paleotetraploid ancestor (Hou et al., 2016; Liu et al., 2016). According to fossil records and plastome studies, the Salicaceae family diverged from its sister family, Lacistemataceae, about 92 million years ago (Davis et al., 2005; Korotkova et al., 2009; Zhang et al., 2018) while *Salix* and *Populus* have diverged about 58-65 million years ago (Hou et al., 2019; Manchester et al., 2006).

1.1.1   Genus *Salix*

Genus *Salix*, commonly named as willows, sallows, and osiers, is the broadest genus in the Salicaceae family with approximately 450 species (Argus, 2010; Newsholme, 1992). Species of *Salix* L. are naturally distributed across the northern hemisphere,

mostly from temperate to arctic regions, and some of them are introduced species to different parts of the world (Lauron-Moreau et al., 2015). Currently, about 65 *Salix* species exist in Europe (Argus, 1997), 103 in North America and Mexico (Argus, 2010), 107 in Russia and northern Asia (Skvortsov, 1999), and 275 in China (Fang et al., 1999). On the other hand, there are 27 *Salix* lineages native to Turkey including four endemic species (Acar et al., 2020; Terzioğlu et al., 2014)

Members of the genus *Salix* have a huge heterogeneity in their sizes such as 30 meters tall trees or only a few centimeters long dwarf shrubs (Stott, 1992), and shape or structure of the leaves and flowers of the catkins inflorescence (Isebrands & Richardson, 2014). Different *Salix* species need different ecological conditions, yet most of them inhabit mesic or hydric habitats which are called riparian and wetland (Skvortsov, 1999). These species have high ecological importance since their ability to be pioneer species for new habitats or niches (Argus, 2010).

1.1.1.1 *Salix alba* L.

S. alba or white willow is a broadleaved and deciduous tree. It has the largest size of its genus, and a fast-growing rate but a relatively short life span of about 20-30 years (CABI, 2013). The leaves of *S. alba* have a narrow and long shape, and the upper side of the leaves has silver-grey color while the lower side has a compact, silky white hair layer (Mitchell et al., 1974). Male flowers bloom on yellow catkins, and female flowers are on greenish-yellow catkins that later become fluffy white to attract insects for pollination. The wind is another common way of dispersal for both pollen and silky-hair-covered, very small seeds of *S. alba* (Mitchell et al., 1974).

S. alba has an allotetraploid genomic structure, and the total chromosome number of the species was detected as 2n = 4x = 76 and n = 2x = 38 in cytological studies of Druskovic (1995) and Khalili et. al, (2012). This phenomenon of allotetraploid in *S. alba* occurs along with disomic inheritance resulting in difficulties in genetic analysis (Barcaccia et al., 2003).

S. alba is well adapted to temperate climates with warm summers including a limited period of drought, and mild winters. Although sandy, calcareous, silty soils promote the optimal growth of it, a wide range of soil types is tolerable for the species throughout its distribution from sea level to 2400 m altitude (Isebrands & Richardson, 2014). Furthermore, water contact is essential for the roots. They might also develop adventitious roots or root suckers and reproduce themselves from these structures as small colonies of clones. Light is the limiting factor of *S. alba* growth since it does not have shadow tolerance while the species can well tolerate the floods more than other flood-tolerant taxa (Houston Durrant et al., 2016).

S. alba has a wide distribution range across Europe, Anatolia (Asia Minor), northern Africa, and western Siberia. It is a highly introduced and neutralized species to the various parts of the world including North America and Australia (Isebrands & Richardson, 2014; Uotila, 2011). Although that results in difficulties in drawing the exact borders of natural habitat for the species together with the many varieties due to selective breeding, it is a well-known fact that *S. alba* L. is native to Europe and Asia (CABI, 2013; POWO, 2021b) (Figure 1.1.).
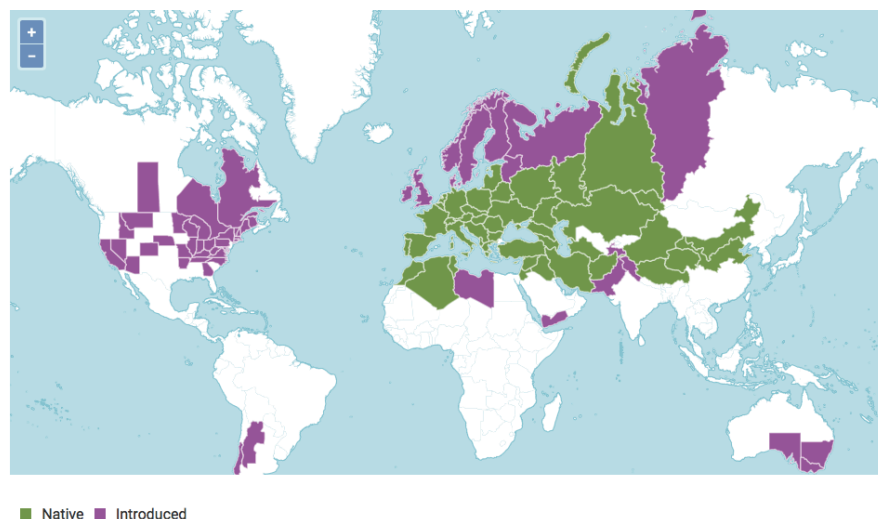
Figure 1.1. Geographic distribution of *S. alba* L. as a native or introduced species (POWO, 2021b)

3

Although there are great variations in climate features among the different geographical regions of Turkey, *S. alba* is naturally distributed in nearly all river basins of Turkey (Davis, 1965) (Figure 1.2.). As a consequence, it is considered the most important willow species in Turkey (Velioğlu & Akgül, 2016).
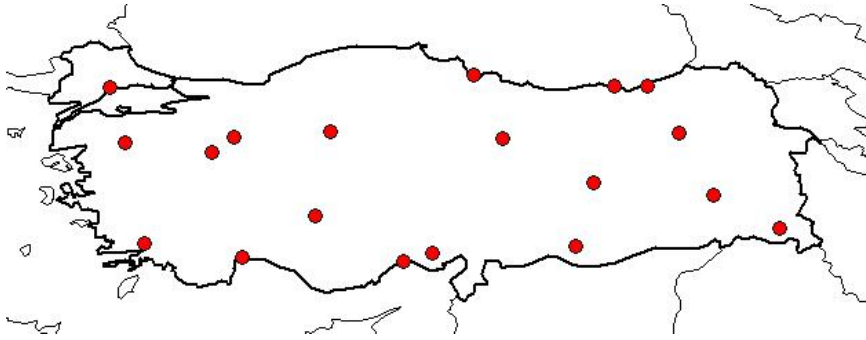
Figure 1.2. Natural distribution of *S. alba* in Turkey (Turkish Plants Data Service (TÜBİVES), 2021)

S. alba has been cultivated for its timber which is used as production of various tools, and for construction purposes (Wilkinson, 1941). In Turkey, it is especially planted for ornamental purposes due to the color of its bark. Phytoremediation, reclamation of the ecosystem, and mitigation of erosion are some of the other highly expanding usage areas of *S. alba* (Ball et al., 2005; Kuzovkina & Quigley, 2005).

1.1.2   Genus *Populus*

Genus *Populus* has 29 known species grouped under six sections regarding the morphologies and crossabilities (Eckenwalder, 1996; OECD, 2006). Many intra- and intersectional hybridizations occur within the genus resulting in a huge diversity, and that makes genetic and phylogenetic analysis difficult. Despite that, it is used as a model genus of molecular genetics, biochemistry, biotechnology, genetic engineering, agronomy, and genomics research on forest trees due to its rapid growth rate, simple vegetative reproduction ability, small genome size, and compliance to genetic transformation (Cervera et al., 2005; Siler et al., 2014).

Species of genus *Populus,* commonly known as poplar, aspen, and cottonwood, are extensively distributed across the temperate and subtropical zones of the Northern Hemisphere with the high capability of adaptation to various ecological conditions (Guleria et al., 2021; Hamzeh et al., 2006). Besides the natural distribution of species of the genus, they have been broadly cultivated for commercial purposes to obtain industrial wood, and for clonal forestry (Hamzeh et al., 2006).

1.1.2.1 *Populus nigra* L.

Populus nigra (European black poplar) belongs to the Aigeiros *Duby* section according to its morphology and nuclear DNA evidence (Eckenwalder, 1996; Hamzeh et al., 2006). It is a fast-growing and deciduous forest tree species that has a large size up to 40 m in height. It has deeply fissured dark brown or black colored bark which the black poplar has been named. It can bear many different leaves in terms of size and shape, but they are usually longer than wider (Mitchell et al., 1974). The fruit type of the species is capsules and they are covered by cottony hair that help the wind dispersal of the seeds to long distances, so gene flow and migration rate are high for the species (Eckenwalder, 1996; Vanden Broeck et al., 2003). Furthermore, *P. nigra* has a diploid chromosome structure with 2n = 38 and n = 19 numbers and can reproduce by both sexual and vegetative propagation naturally (Cervera et al., 2005).

P. nigra has a wide natural distribution range including Europe, Asia, and northern Africa, and naturalized distribution in North and South America, Australia, New Zealand, and China together with the cultivation in some parts of India (CABI, 2013; de Rigo et al., 2016). Humankind introduced the species to various places of the world for several reasons including economic and ornamental purposes. It currently occurs in almost all temperate regions of the world (CABI, 2013; Isebrands & Richardson, 2014; POWO, 2021a) (Figure 1.3.).
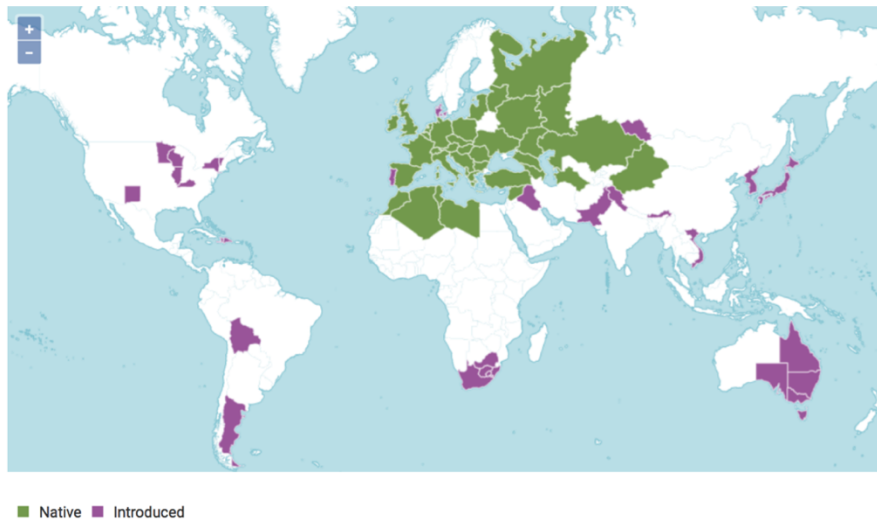
Figure 1.3. Geographic distribution of *P. nigra* L. as a native or introduced species (POWO, 2021a)

P. nigra is one of the widespread tree species for central and eastern Turkey, and is naturally distributed in some parts of Turkey, but currently different varieties of it can grow as an introduced species in almost all river basins in Turkey. (Velioğlu & Akgül, 2016) (Figure 1.4.).

Figure 1.4. Natural distribution of *P. nigra* in Turkey (Ciftci & Kaya, 2019)

P. nigra is considered a highly valuable species in terms of both the ecology and economy of Turkey. It is a keystone species and biodiversity center for riparian ecosystems (Siler et al., 2014; Tognetti et al., 2013). It can grow on floodplains, tolerate high water levels and high summer temperatures yet it is susceptible to shade

and drought (CABI, 2013; OECD, 2006; Siler et al., 2014). It can be used to restore the ecosystem and recolonize the sites that are affected by disturbances like fire or floods (Imbert & Lefevre, 2003). Moreover, regulation of microclimates, moderation of pollutions, management of floods and water quality, enhancement of biological diversity in agricultural areas, stabilization of soil against erosion are the other ecological significances of the species (Norris et al., 2008; Tognetti et al., 2013). On the other hand, the wood of the black poplar is an economically important raw material that is used for furniture, hardwood flooring, pulp, and paper productions (Cottrell, 2004; Siler et al., 2014; Tognetti et al., 2013).

The threats and population decline of the species, especially caused by human activities, show an accelerating trend. Habitat fragmentation and degradation, genetic diversity loss in wild populations due to the plant breeding activities, genetic pollution by gene flow from the cultivated trees and hybrids to the wild individuals are important threats on *P. nigra* that should be scrutinized (CABI, 2013; OECD, 2006; Vanden Broeck et al., 2004).

1.2 Habitat Fragmentation

Habitat fragmentation is the modifications of landscapes that negatively affect biodiversity. It usually occurs in former large forests and brings about a great difference between the patches of vegetation and their surroundings (Chamorro et al., 2015). Fragmentation of landscapes may have some indirect negative effects on species such as biological, behavioral, and intra- and interspecific interactional disturbances or alterations in addition to the decreasing the size of the habitat and declining of species (Fischer et al., 2007). Habitat fragmentation is an ecologically destructive consequence of mainly human activities such as urbanization, agriculture, and building highways and roads. Fragmentation is the contrast of connectivity, so increasing the connectivity between the fragmented landscapes by creating corridors, buffers, and steppingstones for the wildlife are suggested to decrease the negative effects of habitat fragmentation. Furthermore, maintaining or

restoring the vegetation which is similar to the native vegetation in large patches and the surrounding matrix, retaining the key species interactions and functional diversity are some of the other management strategies to mitigate damaging impacts of the fragmentation (Bennett, 2003; Fischer et al., 2006; Huxel et al., 1999). Although river systems are naturally fragmented by several natural agents, human actions like dam construction, road building, and species introductions further divide these habitats into smaller patches (Fuller et al., 2015). That fragmentations of river systems lead to a loss in both animal and plant diversities. Riparian plant species like willow and poplar are affected by habitat fragmentations on river ecosystems, and these species together with their natural riparian ecosystems need to be conserved due to their ecologic and economic importance (de Vries, 2001).

1.3   Microsatellite Markers and Population Genetics

Microsatellites, also named simple sequence repeats (SSR) and short tandem repeats (STR), are repetitive DNA regions of both prokaryotes and eukaryotes with 1 to 10 nucleotides, and they are extensively studied in plant genetics research (Vieira et al., 2016). They are broadly present in the genome, mostly euchromatin regions of eukaryotes, in both coding and non-coding nuclear DNA and organellar DNA (Pérez-Jiménez et al., 2013; Phumichai et al., 2015). Polymorphisms can occur in microsatellite regions of DNA as a result of insertion or deletion mutations. Therefore, different alleles can be found at a microsatellite locus, and detection of different alleles for a locus gives more information about the genetics of the population (Vieira et al., 2016). To detect the presence of a particular microsatellite region in the DNA of an individual, and the alleles of that region, microsatellite markers are developed and used. These markers can be utilized for parental tests, genome mapping, and population genetics research since their special properties include hyper viability, multi-allelic character, co-dominant inheritance, broad genetic distribution, and chromosomal-specific residence (Parida et al., 2009).

CHAPTER 2

JUSTIFICATION AND OBJECTIVES OF STUDY

The major objective of the study is to reveal the impact of habitat fragmentation on genetic structures and diversities of two economically and ecologically important species of the Salicaceae family, namely *Salix alba* and *Populus nigra. P. nigra* is widely used for timber, pulp, and paper productions while *S. alba* is a more ornamentally cultivated species in Turkey. Both species have a great potential for biomass production to obtain bioenergy. Also, they are both pioneer species of riparian ecosystems together with flood tolerance and easy reproduction capability. Thus, they can be used to restore the ecosystem in disturbed sites, stabilize the soil against erosion, and for phytoremediation of soil. However, human-related threats and the population decline of these species have been accelerating in Turkey. To achieve the effects of these human-mediated actions and habitat fragmentations on these two species, nuclear microsatellite markers were used by comparing the populations from the Kızılırmak river basin, representing a highly fragmented habitat, and Melendiz river basin in Ihlara valley, exemplifying a protected habitat.

The study encompasses some specific objectives that are:

- Assessing genetic diversity and patterns, genetic and structural differences among the populations of *S. alba* and *P. nigra* in Kızılırmak and Melendiz river basins,
- Examination of gene flow and magnitude of migrations among two rivers for both *S. alba* and *P. nigra*,

9

- Understanding human impact on *S. alba* and *P. nigra* populations in the scope of habitat fragmentation status of two rivers,
- Providing information on genetic data for further breeding, genetic resource management, conservation studies and practices for both *S. alba* and *P. nigra*.

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Materials

Plant materials of the study were collected from the Kızılırmak River basin and the Melendiz Stream basin in Turkey within the scope of a project, supported by the Scientific and Technical Research Council of Turkey (TUBİTAK), with the project number of TOVAG 213O154, and the name of "Molecular Phylogeny of Turkish *Salix* L. species and genetic characterization of two economically valuable willow species (*Salix alba* and *Salix excelsa*) for tree breeding purposes". Kızılırmak River, which is the longest river in Turkey, represents a highly fragmented river ecosystem while Melendiz Stream in Ihlara Valley is protected from habitat fragmentation. 132 *S. alba* and 84 *P. nigra* genotype samples coming from 4 different populations of Kızılırmak River, and 29 *S. alba* and 25 *P. nigra* genotype samples from one population of Ihlara Valley (Melendiz Stream) were used as the plant materials. Samples were obtained as leaves from randomly chosen willow and poplar trees with the consideration of a 200 m minimum distance to avoid the interference of clonal reproduction for each population. The leaf samples collected from each genotype (individual tree) were kept in separate ziplock bags with silica gels to preserve the plant material from degradation until the DNA isolation process of laboratory work. The detailed location information of the studied populations and the number of samples from the populations were provided in Figure 3.1. and Table 3.1. Also, detailed information on the studied populations across the Kızılırmak and Ihlara river systems was given in Appendix A.

Table 3.1. Information on studied *S. alba* and *P. nigra* populations

River	Location	Population ID		Number of individuals		Altitude (m)	Latitude (N)	Longitude (E)
		S. alba	*P. nigra*	*S. alba*	*P. nigra*			
Kızılırmak	Kayseri/Ürgüp	S-KIZUP-KAY	P-KIZUP-KAY	33	20	789-1113	38°49'55.2"	35°13'30.7"
							38°42'42.8	34°40'28.9"
	Kırşehir	S-KIZMID-KIR	P-KIZMID-KIR	32	22	640-816	40°05'16.1"	33°29'10.3"
							39°36'54.7"	33°26'06.7"
	Kırıkkale	S-KIZMID-KRK	P-KIZMID-KRK	52	28	730-1269	39°41'30.1"	34°59'18.6"
							38°13'45.7"	32°58'40.4"
	Çorum	S-KIZDOWN-COR	P-KIZDOWN-COR	15	14	358-424	41°05'49.6"	35°45'26.3"
							41°00'03.6"	34°25'04.8"
Sub Total				132	84			
Melendiz (Ihlara)	Ihlara/Nevşehir	S-IHR-NEV	P-IHR-NEV	29	25	1102	38°16'46.2"	34°16'37.4"
							38°14'38.6"	34°18'32.4"
Total				161	109			

12
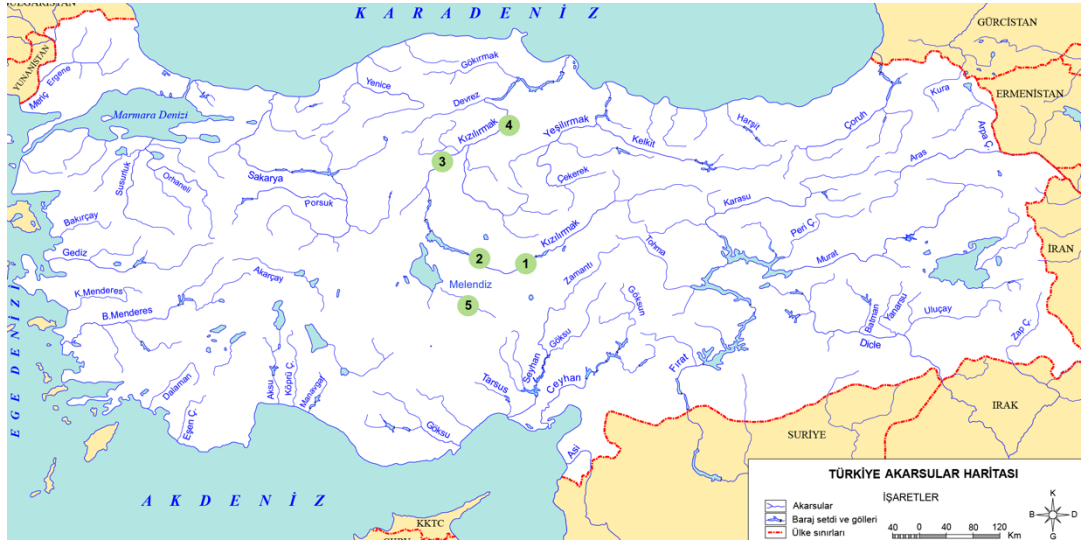
Figure 3.1. Map of the locations of studied populations with the Kızılırmak populations represented by numbers 1-4 and the Ihlara population represented by number 5 (Map edited from cografyaharita.com (Saygılı, 2015)).

3.2 Molecular Studies

3.2.1 DNA Isolation

Leaf samples were treated with liquid nitrogen (-196°C), and ground by sterile mortar and pestle. Then, the genomic DNA isolation procedure for each sample was performed using a modified version of the CTAB extraction protocol (Doyle, 1991). The protocol with the modifications and buffers, chemicals, and equipment used for the isolation were given in detail in Appendix B and Appendix C. To determine the quality, purity, and concentration of the isolated DNA samples, they were measured individually by using NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific, USA). The isolated DNA samples were diluted by their measured concentrations to reach 20 ng/µl template concentration to be used in further PCR amplification.

3.2.2 Microsatellite Loci Amplification by PCR

To investigate the impact of habitat fragmentation on genetic structure and be able to make a comparison between two species (*S. alba* and *P. nigra*) of the Salicaceae family, several microsatellite loci that are possibly common to these two species were tried to be selected from those are originally developed for different *Populus* species. Four of them were already studied by Degirmenci et al. (2019) and Ciftci et al. (2019), and subsets of these data were used for the study. Then, four more nuclear microsatellite markers were detected to be common to both species after several PCR trials of *S. alba* template DNA samples with different microsatellite primers of *Populus* species. Information of these microsatellite loci and the primers that are used were provided in Table 3.2

The chosen primers were optimized for *S. alba* DNA templates by using various amounts of PCR ingredients (5x HOT FIREPol® Blend Master Mix Ready to Load (Solis BioDyne, Tartu, Estonia), which included 15mM $MgCl_2$, forward and reverse primers, diluted DNA template, and dH_2O) with changing PCR program conditions. The optimized PCR ingredients and program conditions for each microsatellite locus were given in Table 3.3. The amplifications were evaluated by loading and running the PCR products on %3 agarose gel. Then, the gel imaging system (Vilbor Lourmat, France) was used to examine the gels under the UV light to check the presence and sizes of amplified PCR product bands.

Table 3.2 Information on microsatellite loci and their primers

	Locus ID	Repeat Motif	Primer Pairs as: Forward Primer (5'– 3') Reverse Primer (3' – 5')	Expected Product Size (bp)	Reference
Primers amplifications from this study	WPMS04	$(GT)_{25}$	Forward: ACACGGGTCTTTTATTCTCT Reverse: TGCCGACATCCTGCGTTCC	275	Van Der Schoot et al., 2000
	WPMS14	$(CGT)_{28}$	Forward: CAGCCGCAGCCACTGAGAAATC Reverse: GCCTGCTGAGAAGACTGCCTTGAC	245	Smulders et al., 2001
	WPMS16	$(GTC)_8$	Forward: CTCGTACTATTTCCGATGATGACC Reverse: AGATTATTAGGTGGGCCAAGGACT	150	
	PMGC14	CTT	Forward: TTCAGAATGTGCATGATGG Reverse: GTGATGATCTCACCGTTTG	210	IPGC SSR Resource* as cited in Liesebach et al., 2010
Primers amplifications from the previous studies**	PMGC2163	GA	Forward: CAATCGAAGGTAAGGTTAGTG Reverse: CGTTGGACATAGATCACACG	220	
	PMGC2709	GA	Forward: ATTGTAATTATTGAACACATGCC Reverse: GTGCAGTTCAGAGTATTGTTG	210	Lin et al., 2009
	PMGC2889	GA	Forward: CCCAAGATCCGATTTTTGGG Reverse: CACAATGTACAAATCGCTGTC	207	
	WPMS18	$(GTG)_{13}$	Forward: CTTCACATAGGACATAGCAGCATC Reverse: CACCAGAGTCATCACCAGTTATTG	245	Smulders et al., 2001

*IPGC SSR Resource: The International *Populus* Genome Consortium derived microsatellite loci from http://www.ornl.gov/sci/ipgc/ssr_resource.htm

** Previous studies: Degirmenci et al. (2019) and Ciftci et al. (2019)

15

Table 3.3 Optimized PCR ingredient compositions and cycling program parameters for the studied microsatellite loci

		Locus ID			
		WPMS04	WPMS14	WPMS16	PMGC14
PCR Ingredients	**dH2O**	10µl	10.5µl	14µl	10µl
	Master Mix	5.5µl	5µl	4µl	5.5µl
	Primers (10µM)	(0.75+0.75) µl	(0.75+0.75) µl	(0.5+0.5) µl	(0.75+0.75) µl
	DNA (20ng/µl)	8µl	8µl	6µl	8µl
	Total	25µl	25µl	25µl	25µl
PCR Cycling Parameters	**Initial Denaturation**	94ºC, 3'	94ºC, 3'	94ºC, 3'	94ºC, 3'
	Denaturation	94ºC, 30"	94ºC, 30"	94ºC, 30"	94ºC, 30"
	Annealing	55ºC, 45"	56ºC, 45"	56ºC, 45"	55ºC, 45"
	Extension	72ºC, 1'	72ºC, 1'	72ºC, 1'	72ºC, 1'
	Final Extension	72ºC, 20'	72ºC, 20'	72ºC, 20'	72ºC, 20'

(Denaturation, Annealing, Extension rows: 35 cycles for WPMS04, 35 cycles for WPMS14, 30 cycles for WPMS16, 35 cycles for PMGC14)

3.2.3   DNA Fragment Analysis

The forward primers used in the study were labeled with three different fluorescent dyes at their 5' ends as given in Table 3.4. When all of the SSR loci were amplified using these primers with all DNA samples, PCR products of them were sent to BM Laboratory Systems Facilities, Ankara to conduct DNA fragment analyses. For the fragmental analysis protocol, Applied Biosystems 3730 XL DNA Analyzer (Applied Biosystems Inc., Foster City, CA, 2006) was used with the GeneScan™ LIZ® labeled 500LIZ internal standard size marker. To determine the relative sizes of fragments for SSR loci, fragments were compared with the standard curve during the analysis. As the result of this analysis, an electropherogram was obtained for each sample, and the Peak Scanner Software 2.0 (Applied Biosystems Inc., Foster City, CA, 2006) was used to detect allele sizes of each locus. An example of the electropherogram image was provided in Figure 3.2.

Table 3.4. SSR loci of primers and their fluorescent dye labels

Locus ID	Fluorescent Dye
WPMS04	TAMRA (Black)
WPMS14	HEX (Green)
WPMS16	6-FAM (Blue)
PMGC14	6-FAM (Blue)
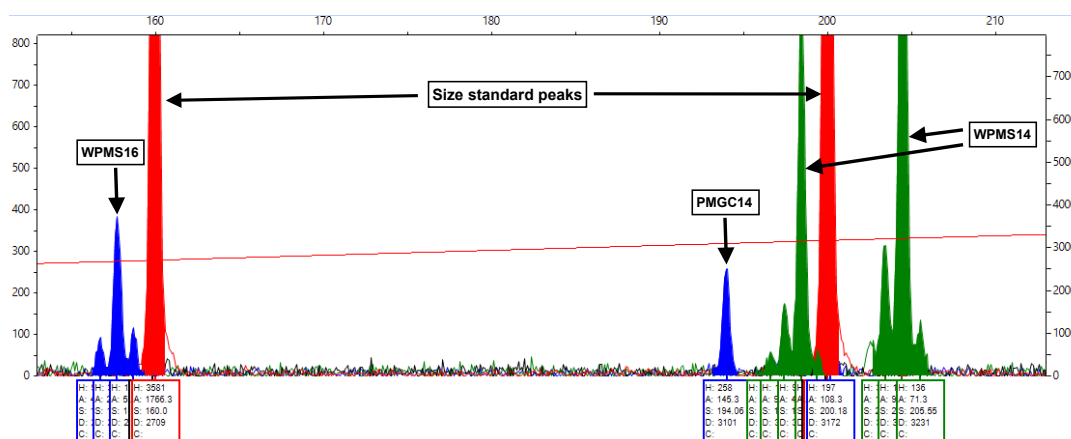
Figure 3.2. Example of an electropherogram image of studied three different microsatellite loci

3.3 Data Assessment

A part of the data used in this study was derived from the subsets of the previous studies. *S. alba* microsatellite data of Kızılırmak populations for PMGC2163, PMGC2709, PMGC2889, and WPMS18 loci were obtained from the study of Degirmenci et al. (2019). Similarly, *P. nigra* microsatellite data of Kızılırmak populations for PMGC2163, PMGC2709, PMGC2889, WPMS18, WPMS04, WPMS14, WPMS16, and PMGC14 loci were acquired from the study of Ciftci et al. (2019). Besides that, the microsatellite data of the Ihlara population of *S. alba* for the same loci as above were utilized from the TOVAG 213O154 project of "Molecular Phylogeny of Turkish *Salix* L. species and genetic characterization of two economically valuable willow species (*Salix alba* and *Salix excelsa*) for tree breeding purposes", and *P. nigra* microsatellite data of the Ihlara population were retrieved

from TOVAG-110O570 project of "Genetic characterization of Turkish Black poplar genetic resources and development of molecular black poplar breeding program". Subsequently, these data were combined with the data from the laboratory work of this study for further genetic structure and population genetics analyses.

3.4   Population Genetics Analyses

3.4.1   Quality Control of Microsatellite Markers and Allele Detection

Genotyping errors, stutter peaks, large allele dropouts were checked, and typography mistakes were fixed. As the first step of analyses, allele numbers and size range were detected via MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004).

3.4.2   Detection of Clonal Reproduction

Clonal reproduction is one of the common characteristics for all species belonging to the Salicaceae family. Therefore, both genus *Salix* and genus *Populus* may reproduce asexually from buds on their roots and shoots. Moreover, they have vegetative reproduction ability via very small fragments like broken branches with shedding zone dispersed by the water, or by whole trees drifted by floods (Karrenberg et al., 2002). For this reason, there is a probability to have clones among the samples although the sampling was carried out with the consideration of the minimum 200-meter distance between the individuals. To avoid the interference of those possible clones on the genetic diversity and structure analyses by generating biased results, it is important to detect the clonal duplications of the samples. GenClone 2.0 software (Arnaud-Haond & Belkhir, 2006) was used to determine the presence of genetically identical clones by using the data of the studied eight microsatellite loci.

3.4.3 Presence of Null Alleles

If an allele of a microsatellite locus is constantly failing to amplify and escape from the detectable levels during PCR amplification, that is the microsatellite null allele which is an undesirable tendency of microsatellite markers (Dakin & Avise, 2004).

Null alleles cause the appearance of a locus as homozygous although it is heterozygous in fact. That leads to biased estimates for genetic parameters and deviations from Hardy-Weinberg equilibrium (Wu et al., 2019). MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004) and Genepop 4.2 (Raymond & Rousset, 1995) software were used together to get more reliable results for null allele frequency estimations for each locus and each population of both *S. alba* and *P. nigra*. Genepop 4.2 (Raymond & Rousset, 1995) software implements Brookfield's (1996) method only while MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004) software carries out both Brookfield (1996) and Chakraborty et al. (1992) methods together for the null allele estimations.

3.4.4 Microsatellite Polymorphism and Genetic Diversity Parameters

After the detection of clones and the presence of null alleles, genetic diversity parameters were determined. By using GenAlEx v6.503 (Peakall & Smouse, 2012), the mean number of alleles (Na), the mean number of effective alleles (Ne), Shannon's Information Index (I), observed heterozygosity (Ho), unbiased expected heterozygosity (uHe), fixation index (F), private alleles and their frequencies were computed for each locus and population of both species individually. Allelic richness (Ar), another important genetic diversity parameter, was calculated by FSTAT v2.9.3.2 (Goudet, 1995). Ar provides an unbiased estimation value of allelic richness in terms of different sampling sizes by standardizing the number of alleles based on the smallest population size.

Polymorphism information content (PIC) was estimated with Cervus v3.0.7 (Kalinovski et al., 2007) for each locus of each species to achieve a better idea

regarding polymorphism levels. Subsequently, probability of identity (PI) values of each locus and percentage of polymorphic loci (%P) of each population were calculated by GenAlEx v6.503 (Peakall & Smouse, 2012) for both species.

To compute the F statistics (F_{IS}, F_{ST}, F_{IT}) together with the exact test of Hardy-Weinberg deviations of each locus, Genepop v4.2 (Raymond & Rousset, 1995) was used for both *S. alba* and *P. nigra* populations. A Markov chain algorithm was set with the parameters as Dememorization number=1000, Number of batches=100, Number of iterations per batch=1000 to obtain unbiased p-values of the exact test (Guo & Thompson, 1992). The number of migrants (Nm) value which implies the degree of gene flow among populations was estimated by using GenAlEx v6.503 (Peakall & Smouse, 2012).

Garza-Williamson Index values (Garza & Williamson, 2001) of all loci and populations of both species were determined to investigate any possible past effective size reductions of populations such as bottleneck events by Arlequin v3.5.1.2 (Excoffier & Lischer, 2010).

To assess the partition of total genetic variation as among two rivers, among populations within rivers and within populations, the analysis of molecular variance (AMOVA) was performed for both species by using Arlequin v3.5.1.2 (Excoffier & Lischer, 2010). The analyses were done based on two different models individually. The first model, the infinite allele model, is based on the number of different alleles (F_{ST}-based) while the second model, the stepwise mutation model, is based on the sum of squared size differences (R_{ST}-based).

3.4.5   Population Genetic Structures

Genetic differentiations between the populations of both *S. alba* and *P. nigra* were explored by generating pairwise F_{ST} matrices and obtaining pairwise F_{ST} values for each species individually. Arlequin v3.5.1.2 (Excoffier & Lischer, 2010) was used to achieve the pairwise F_{ST} values and the corresponding Nm values.

To visualize the genetic differentiation among the populations of both species, principal coordinates analysis (PCoA) was carried out by using pairwise F_{ST} values and based on the covariance matrix with data standardization via GenAlEx v6.503 (Peakall & Smouse, 2012).

The phenetic relationships among the populations of both *S. alba* and *P. nigra* independently, and also *en masse* were revealed by constructing different phenograms by using GDA (Genetic Data Analysis) software (Lewis & Zaykin, 2001) together with PAUP* v4.0 (Swofford, 2003). Unweighted Pair-Group Method with Arithmetic Averaging (UPGMA) algorithm was employed as the distance method of hierarchical clustering of populations.

Genetic structure and clustering patterns of *S. alba* and *P. nigra* populations were determined with the help of STRUCTURE v2.3.4 (Pritchard et al., 2000) software by a Bayesian algorithm to distribute the individuals into clusters to determine the true number of clusters for each species. Parameters of the Bayesian algorithm were set as 50,000 burn-ins and 250,000 Markov chain Monte Carlo (MCMC) iterations, and the admixture model was selected as the ancestry model of simulations. Then, all populations together of each species were tested with the possible cluster numbers (K) of K=1 to K=5. Each K value was tested with 10 iterations. Moreover, these simulations were performed with and without prior information about the populations individually.

The run data obtained from STRUCTURE v2.3.4 (Pritchard et al., 2000) were processed by using a web-based tool, namely Structure Harvester (Earl & VonHoldt, 2012) to estimate true K values of populations according to the Evanno method (Evanno et al., 2005). After the decision of true K values, runs of these K values were merged by CLUMPP (Jakobsson & Rosenberg, 2007) for the identification of the ideal alignment of the replicates. Afterward, Pophelper (Francis, 2017) was used to visualize the structural data with the graphical representations of clustering patterns.

CHAPTER 4

RESULTS

4.1   Allele Detection, Quality Control and Polymorphism of Microsatellites

As the first step of analyses, alleles of the microsatellite markers were detected manually by using Peak Scanner Software 2.0 (Applied Biosystems Inc., Foster City, CA, 2006), and they were verified via MICRO-CHECKER v2.2.3 (Van Oosterhout et al., 2004) by correcting any genotyping errors and typography mistakes.

The observed alleles for eight SSR loci for *S. alba* and *P. nigra* populations were provided in Table 4.1. and Table 4.2. respectively. According to the results, all loci were found to be polymorphic for all populations of both species. In addition, the least variable locus is WPMS16 with 6 alleles and the most variable one is PMGC2709 with 20 alleles for *S. alba* populations while the least and the most variable loci are WPMS16 with 7 alleles and PMGC2163 with 17 alleles respectively for *P. nigra* populations. There are several private alleles were detected for each locus of the populations and they were also indicated as bold in the tables.

Table 4.1. Observed alleles and allelic size range of each microsatellite locus for *S. alba* populations (Private alleles are shown bold)

Locus ID	Size Range	Number of Alleles	Observed Alleles (bp)
WPMS16	152-176	6	**152**, 158, **164**, 167, 173, **176**
PMGC14	182-233	16	182, 185, **188**, 191, 194, 197, 200, 203, 206, 209, 212, 215, 218, **224**, 227, **233**
WPMS14	189-222	9	189, 192, 198, 201, 204, 210, 216, **219**, 222
WPMS04	312-382	17	312, 316, 320, 324, 328, **330**, 334, 340, 344, 350, 354, 358, 360, 364, 370, 376, **382**
PMGC2709	166-218	20	**166**, 172, 180, 184, 186, 188, 190, 192, 194, 196, **198**, **200**, 202, 204, 208, 210, 212, **214**, 216, 218
PMGC2889	174-222	18	174, **178**, 182, 184, 186, 188, 190, 192, 194, 198, **200**, 202, 204, 206, 208, **210**, 212, **222**
WPMS18	211-232	7	211, 214, **217**, 220, **223**, 226, 232
PMGC2163	186-226	14	**186**, 188, 194, 198, 200, 204, 206, 208, 210, 212, 214, 216, 224, **226**

Table 4.2. Observed alleles and allelic size range of each microsatellite locus for *P. nigra* populations (Private alleles are shown bold)

Locus ID	Size Range	Number of Alleles	Observed Alleles (bp)
WPMS16	135-159	7	**135**, 141, 144, 150, 153, **156**, **159**
PMGC14	189-225	12	**189**, 192, 195, 198, 201, 204, 207, 210, **213**, **216**, 219, **225**
WPMS14	210-261	9	210, **216**, **225**, 231, 237, 243, **249**, 252, 261
WPMS04	246-286	8	246, 248, **254**, **256**, 260, **264**, 274, **286**
PMGC2709	190-216	9	190, 194, 198, **200**, **202**, 204, **208**, **210**, **216**
PMGC2889	180-219	9	**180**, 184, 192, 198, 200, 202, 212, **216**, **218**
WPMS18	225-261	9	225, 228, 231, 234, 237, **246**, 249, **252**, **261**
PMGC2163	186-268	17	**186**, **192**, **198**, **206**, **210**, 216, **220**, **222**, 224, 228, 234, 240, **242**, 246, **252**, 256, **268**

4.2 Detection of Clones and Null Allele Presence

The molecular data were analyzed by GenClone v2.0 software (Arnaud-Haond & Belkhir, 2007) to detect any genetically identical individual among the samples. As the result of the analysis, it was determined that there is no clone within samples.

According to the MICRO-CHECKER v2.2.3 (Van Oosterhout et al., 2004) results given in Table 4.3. and Table 4.4., null alleles might be present with slightly higher frequencies at WPMS18 locus in S-KIZMID-KIR and S-KIZMID-KRK populations, at WPMS04 locus in S-KIZMID-KIR, S-KIZMID-KRK, and S-IHR-NEV populations, and PMGC2709 locus in the S-IHR-NEV population of *S. alba*. Since that is limited to only these particular populations and they do not show large allele dropout or stuttering, further analyses were performed both including and excluding the possible null alleles. The difference between the statistical results was not significant, so no null alleles were assumed for the population genetics analyses of the study.

Table 4.3. Null Allele frequency estimations for each locus for *S. alba* populations

| Locus ID | S-KIZUP-KAY | | S-KIZMID-KIR | | S-KIZMID-KRK | | S-KIZDOWN-COR | | S-IHR-NEV | |
	B	C	B	C	B	C	B	C	B	C
WPMS16	0.00	-0.01	0.00	-0.01	0.00	-0.01	0.00	-0.02	0.00	-0.02
PMGC14	0.07	0.09	0.01	0.02	0.00	-0.05	0.01	0.01	0.00	-0.01
WPMS14	0.00	-0.18	0.00	-0.17	0.00	-0.09	0.00	-0.08	0.01	0.01
WPMS04	0.00	-0.01	0.07*	0.09*	0.09*	0.10*	0.09	0.11	0.14*	0.18*
PMGC2709	0.00	-0.02	0.04	0.05	0.03	0.03	0.00	-0.04	0.52*	0.23*
PMGC2889	0.00	-0.02	0.00	-0.08	0.02	-0.08	0.00	-0.14	0.34	-0.01
WPMS18	0.05	0.14	0.20*	0.41*	0.07*	0.23*	0.26	-0.02	0.00	-0.15
PMGC2163	0.00	-0.04	0.00	-0.14	0.00	-0.18	0.00	-0.20	0.00	-0.17

(**B**: Brookfield method value, **C**: Chakraborty method value) *Possible null allele presenting loci

Table 4.4. Null Allele frequency estimations for each locus for *P. nigra* populations

	P-KIZUP-KAY		P-KIZMID-KIR		P-KIZMID-KRK		P-KIZDOWN-COR		P-IHR-NEV	
Locus ID	**B**	**C**	**B**	**C**	**B**	**C**	**B**	**C**	**B**	**C**
WPMS16	0.00	-0.31	0.00	-0.27	0.00	-0.25	0.00	-0.31	0.08	-0.20
PMGC14	0.00	-0.23	0.00	-0.25	0.00	-0.17	0.00	-0.28	0.05	-0.19
WPMS14	0.00	-0.17	0.00	-0.25	0.00	-0.15	0.00	-0.24	0.04	-0.25
WPMS04	0.00	-0.01	0.01	0.01	0.00	-0.03	0.00	-0.04	0.28	0.21
PMGC2709	0.00	-0.22	0.00	-0.21	0.04	-0.18	0.00	-0.28	0.13	-0.23
PMGC2889	0.00	-0.22	0.00	-0.26	0.00	-0.05	0.00	-0.28	0.00	-0.16
WPMS18	0.00	-0.22	0.00	-0.21	0.00	-0.02	0.00	-0.28	0.04	-0.25
PMGC2163	0.00	-0.21	0.00	-0.25	0.06	-0.12	0.00	-0.28	0.07	-0.05

(**B**: Brookfield method value, **C**: Chakraborty method value)

4.3   Genetic Diversity Factors

4.3.1   Genetic Diversity Parameters of Microsatellites

The genetic diversity parameters of eight microsatellite loci for *S. alba* populations were given in Table 4.5. and for *P. nigra* populations in Table 4.6. The mean number of alleles per locus (Na) varies from 3.00±0.32 (WPMS16) to 12.80±1.28 (WPMS04) for *S. alba*, and from 3.40±0.51 (WPMS16) to 6.20±1.24 (PMGC2163) for *P. nigra*. For *S. alba* and *P. nigra* species, the range of the number of effective alleles (Ne) is between 1.08±0.02 (WPMS16) and 6.85±0.78 (WPMS04), and between 1.46±0.09 (WPMS04) and 3.06±0.51 (PMGC2163) respectively. Likewise, Shannon's Information Index (I), which indicates a measure of genetic diversity, changes over loci and populations, has the mean value of 1.36±0.11 and 1.04±0.05 for *S. alba* and *P. nigra* populations respectively. The means of observed heterozygosity (Ho) values are higher than the means of unbiased expected heterozygosity (uHe) values for both species. The mean Ho values were 0.63±0.05 and 0.87±0.04 for *S. alba* and *P. nigra*, respectively while the mean uHe values were detected as 0.61±0.05 and 0.59±0.02 with the same order. However, these values

were uniquely found as almost equal at WPMS16 locus of *S. alba* populations while other loci were showing both higher and lower observed heterozygosity than the expected ones. For *P. nigra*, all loci have higher Ho values than the uHe except WPMS04. The higher Ho than uHe points out the excess of heterozygotes and receives a negative fixation index (F) value while the lower Ho than uHe indicates heterozygote deficiency and gets a positive fixation index (F) value. WPMS04 has the highest allelic richness (Ar) value (10.732) and WPMS16 has the lowest (1.881) for *S. alba* populations while PMGC2163 has the highest (7.430) and WPMS04 has the lowest (3.402) value for *P. nigra* populations. Probability of identity (PI) values are varying between 0.024 at PMGC2709 locus and 0.861 at WPMS16 locus of *S. alba* populations, and between 0.091 at PMGC14 and 0.477 at WPMS04 loci of *P. nigra* populations. Polymorphism Information Content (PIC) values express the grades of the polymorphism among loci. PIC values of the studied eight loci for *S. alba* populations have a 0.607 mean value, ranging from 0.073 (WPMS16) to 0.875 (PMGC2709). For *P. nigra* populations, the mean PIC value was found to be 0.577 and it ranged between 0.303 (WPMS04) and 0.688 (PMGC2163).

F statistics analyses of eight microsatellite loci were performed for all populations of both species. The results of these F statistics analyses were provided in Table 4.7. Although means of F_{IS} values are negative for *S. alba* and *P. nigra*, there are also some positive F_{IS} values were detected for some loci such as PMGC14, WPMS04, PMGC2709, and WPMS18 for *S. alba* populations; WPMS04 for *P. nigra*. Negative F_{IS} values indicate an excess of heterozygosity in the population possibly caused by outbreeding while positive F_{IS} values have the meaning of deficiency in heterozygosity, or inbreeding. Mean F_{ST} values were found as positive for both species. *S. alba* populations had the mean F_{ST} value of 0.0373 and it varied from -0.0064 (WPMS16) to 0.0950 (WPMS18). The mean F_{ST} value for *P. nigra* populations was 0.0787 and ranged from 0.0041 (WPMS14) to 0.1880 (PMGC14). The maximum Nm value is 31.6358 at WPMS16 in *S. alba* and 19.8743 at WPMS14 in *P. nigra* while the minimum values of the populations of *S. alba* and *P. nigra*, respectively are 2.1303 at WPMS18 and 1.3297 at PMGC14.

Table 4.5. Genetic diversity parameters of the eight microsatellite loci for *S. alba* populations

Locus ID	N	Na	Ne	I	Ho	uHe	F	Ar	PI	PIC
WPMS16	32.20±5.91	3.00±0.32	1.08±0.02	0.19±0.03	0.08±0.02	0.08±0.01	-0.03±0.01	1.881	0.861	0.073
PMGC14	32.20±5.91	10.00±1.14	4.71±0.36	1.78±0.09	0.77±0.04	0.80±0.02	0.02±0.05	7.451	0.061	0.787
WPMS14	32.20±5.91	4.80±1.07	2.18±0.20	0.93±0.12	0.66±0.10	0.53±0.05	-0.24±0.09	3.723	0.275	0.477
WPMS04	32.20±5.91	12.80±1.28	6.85±0.78	2.17±0.10	0.70±0.03	0.86±0.02	0.17±0.05	10.732	0.029	0.856
PMGC2709	30.60±6.33	11.20±1.53	6.32±0.52	2.03±0.10	0.77±0.06	0.85±0.01	0.08±0.08	10.423	0.024	0.875
PMGC2889	30.60±6.09	10.40±0.93	5.56±0.48	1.93±0.09	0.93±0.03	0.83±0.02	-0.14±0.06	8.885	0.044	0.820
WPMS18	32.00±6.06	3.80±0.73	1.68±0.29	0.67±0.20	0.29±0.14	0.34±0.10	0.16±0.16	4.077	0.398	0.369
PMGC2163	32.20±5.91	7.20±0.80	2.50±0.20	1.22±0.05	0.81±0.09	0.60±0.04	-0.36±0.08	6.437	0.166	0.598
Grand Mean	31.78±1.93	7.90±0.65	3.86±0.36	1.36±0.11	0.63±0.05	0.61±0.05	-0.04±0.04	-		0.607

N: Sample size, Na: mean allele number, Ne: effective number of alleles, I: Shannon's Information Index, Ho: observed heterozygosity, uHe: unbiased expected heterozygosity, F: inbreeding coefficient, Ar: allelic richness, PI: Probability of Identity, PIC: polymorphism information content

Table 4.6. Genetic diversity parameters of the eight microsatellite loci for *P. nigra* populations

Locus ID	N	Na	Ne	I	Ho	uHe	F	Ar	PI	PIC
WPMS16	21.40±2.27	3.40±0.51	2.28±0.19	0.87±0.09	0.95±0.02	0.57±0.03	-0.74±0.07	3.621	0.261	0.496
PMGC14	21.60±2.32	5.60±0.93	2.72±0.15	1.18±0.09	0.98±0.01	0.64±0.02	-0.58±0.07	6.768	0.091	0.726
WPMS14	21.60±2.32	5.00±0.45	2.78±0.15	1.16±0.05	0.98±0.02	0.65±0.02	-0.54±0.07	4.303	0.191	0.581
WPMS04	21.40±2.27	3.40±0.68	1.46±0.09	0.57±0.08	0.30±0.06	0.31±0.05	0.04±0.08	3.402	0.477	0.303
PMGC2709	21.00±2.10	4.40±0.40	2.62±0.15	1.08±0.07	0.96±0.01	0.63±0.02	-0.58±0.05	4.001	0.208	0.559
PMGC2889	21.80±2.38	4.80±0.92	2.86±0.27	1.16±0.11	0.95±0.04	0.66±0.03	-0.51±0.12	5.231	0.158	0.624
WPMS18	21.60±2.32	4.80±0.49	2.49±0.09	1.06±0.05	0.90±0.07	0.61±0.01	-0.52±0.13	5.468	0.146	0.641
PMGC2163	21.40±2.18	6.20±1.24	3.06±0.51	1.26±0.16	0.92±0.04	0.66±0.04	-0.46±0.12	7.430	0.110	0.688
Grand Mean	21.48±0.73	4.70±0.28	2.53±0.11	1.04±0.05	0.87±0.04	0.59±0.02	-0.48±0.05	-		0.577

N: Sample size, Na: mean allele number, Ne: effective number of alleles, I: Shannon's Information Index, Ho: observed heterozygosity, uHe: unbiased expected heterozygosity, F: inbreeding coefficient, Ar: allelic richness, PI: Probability of Identity, PIC: polymorphism information content

Table 4.7. F-Statistics and Nm estimations for each locus over the populations of *S. alba* and *P. nigra*

						Locus ID				
		WPMS16	**PMGC14**	**WPMS14**	**WPMS04**	**PMGC2709**	**PMGC2889**	**WPMS18**	**PMGC2163**	**Mean**
S. alba Populations	F_{IS}	-0.0173	0.0175	-0.2635***	0.1820***	0.0897***	-0.1153***	0.1969***	-0.3485***	-0.0248***
	F_{IT}	-0.0238	0.0300	-0.1503	0.1885	0.1247	-0.1015	0.2733	-0.2655	0.0135
	F_{ST}	-0.0064	0.0127	0.0896	0.0079	0.0384	0.0124	0.0950	0.0616	0.0373
	N_M	31.6358	6.8842	2.1375	9.6824	4.1938	9.3696	2.1303	3.8640	8.7372
P. nigra Populations	F_{IS}	-0.6916***	-0.5278***	-0.5020***	0.0452	-0.5314***	-0.4175***	-0.4496***	-0.3772***	-0.4582***
	F_{IT}	-0.5951	-0.2406	-0.4958	0.0670	-0.5249	-0.3628	-0.2272	-0.2439	-0.3434
	F_{ST}	0.0570	0.1880	0.0041	0.0229	0.0042	0.0386	0.1534	0.0968	0.0787
	N_M	4.8672	1.3297	19.8743	6.1745	18.3227	5.7837	1.7196	2.5891	7.5826

F_{IS}: Inbreeding Coefficient Within Individuals; F_{IT}: Inbreeding Coefficient Within Total Population; F_{ST}: Inbreeding Coefficient Within Populations; N_M: Number of Migrants (***:p<0.001,**:p<0.01,*:p<0.05)

4.3.2   Genetic Diversity Among Populations of Two Species

Detected private alleles and their frequencies for each locus among the populations of *S. alba* and *P. nigra* were provided in Appendix D. The graphical representations of allelic patterns across populations of *S. alba* and *P. nigra* were given in Appendix E. Also, Table 4.8. contains the genetic diversity parameters of both *S. alba* and *P. nigra* populations.

At least one private allele was detected for all populations of both species at different or the same loci. The least number of private allele of *S. alba* belongs to the downstream population of Kızılırmak (Çorum) and the highest numbers of private allele are found in the Kırıkkale middle population of Kızılırmak and Ihlara population. In *P. nigra* populations, upstream (Kayseri) and downstream (Çorum) populations of Kızılırmak share the minimum number of private allele while the Ihlara population has the maximum number of private allele. The mean number of different alleles (Na) ranged from 5.25±0.88 (S-KIZDOWN-COR) to 9.38±1.51 (S-KIZUP-KAY) in *S. alba* populations. Similarly, Na varied between 3.50±0.19 (P-KIZDOWN-COR) and 6.50±0.65 (P-KIZMID-KRK) in *P. nigra* populations. On the other hand, the S-IHR-NEV population of *S. alba* had the highest mean value for the number of effective alleles (Ne) as 4.42±1.02, and the S-KIZDOWN-COR population had the lowest with 3.19±0.60. However, means of Ne had closer values among *P. nigra* populations varying from 2.14±0.14 (P-KIZDOWN-COR) to 2.84±0.37 (P-IHR-NEV).

The minimum mean values of Shannon's Information Index (I) were found in the downstream population of Kızılırmak for both *S. alba* and *P. nigra* with the values of 1.17±0.24 for *S. alba* and 0.85±0.07 for *P. nigra* while the maximum I mean values were detected in the S-IHR-NEV population of *S. alba* with 1.45±0.26 and P-KIZMID-KRK population of *P. nigra* with 1.22±0.09. Observed heterozygosity (Ho) had the highest value in one of the middle populations of Kızılırmak (S-KIZMID-KRK) and the lowest in the Kızılırmak upstream population (S-KIZUP-

KAY) of *S. alba*. On the contrary, the highest and the lowest Ho values of *P. nigra* were estimated in the upstream population (P-KIZUP-KAY) and one of the middle populations (P-KIZMID-KRK) of Kızılırmak. When the observed heterozygosity levels of the two species were compared, it was found that *P. nigra* showed higher heterozygosity for all populations than *S. alba*. Kızılırmak downstream (S-KIZDOWN-COR) population of *S. alba* had the minimum value for unbiased expected heterozygosity (uHe) while the Ihlara (S-IHR-NEV) population had the maximum uHe value. For *P. nigra* populations, P-KIZDOWN-COR had the minimum and P-KIZMID-KRK had the maximum uHe value. Moreover, all studied loci were found to be 100% polymorphic for all populations of both species. The degree of differentiation between Kızılırmak populations of *S. alba* was found as F_{ST}=0.0156, and for overall differentiation among all populations of *S. alba* was 0.0538. Likewise, differentiation among Kızılırmak populations of *P. nigra* was 0.0179 while overall differentiation among all populations of *P. nigra* was high (F_{ST}=0.1352) (Table 4.8.).

Garza-Williamson index (M) values are used to estimate whether the populations faced any size reduction in the past. It reveals the ratio of the mean number of alleles to allelic size range since allelic size range shows a slower decrease than the number of alleles during the bottleneck event of a population (Garza & Williamson, 2001). If the M value of a population is lower than the 0.68 critical value, the bottleneck event can be interpreted for the population. On the other hand, if the M value is higher than 0.80, it implies no reduction of effective population size. According to Table 4.8., M values ranged between 0.226±0.072 (S-KIZDOWN-COR) and 0.264±0.064 (S-KIZUP-KAY) for *S. alba* populations, and between 0.162±0.057 (P-IHR-NEV) and 0.220±0.074 (P-KIZMID-KRK) for *P. nigra* populations. In addition, Garza-Williamson indexes at different loci for each population of *S. alba* and *P. nigra* were given as bar graphs in Figure 4.1. and Figure 4.2., respectively. Considering that all the populations and loci with M values were lower than the 0.68 critical value, it can be made the inference that populations had undergone bottleneck events.

Table 4.8. Genetic diversity parameters of *S. alba* and *P. nigra* populations

	Population	N	Na	Ne	I	%P	G-W Index (M)	Ho	uHe	F	F_{ST}
S. alba Populations	S-KIZUP-KAY	33.00±0.00	9.38±1.51	3.41±0.66	1.35±0.26	100.00%	0.264±0.064	0.60±0.11	0.59±0.10	-0.03±0.07	0.01560*** / 0.05381***
	S-KIZMID-KIR	32.00±0.00	8.25±1.62	4.08±0.86	1.43±0.27	100.00%	0.263±0.046	0.63±0.11	0.64±0.10	-0.01±0.11	
	S-KIZMID-KRK	51.88±0.13	9.25±1.76	4.20±0.98	1.42±0.29	100.00%	0.241±0.055	0.65±0.12	0.61±0.11	-0.04±0.09	
	S-KIZDOWN-COR	14.88±0.13	5.25±0.88	3.19±0.60	1.17±0.24	100.00%	0.226±0.072	0.63±0.13	0.57±0.11	-0.12±0.08	
	S-IHR-NEV	27.13±1.23	7.38±1.12	4.42±1.02	1.45±0.26	100.00%	0.234±0.083	0.62±0.10	0.65±0.10	-0.02±0.10	
	Mean	31.78±1.93	7.90±0.65	3.86±0.36	1.36±0.11	100.00%	0.246±0.064	0.63±0.05	0.61±0.05	-0.04±0.04	
P. nigra Populations	P-KIZUP-KAY	20.00±0.00	3.63±0.38	2.57±0.17	1.02±0.08	100.00%	0.197±0.105	0.91±0.07	0.61±0.04	-0.52±0.09	0.01792* / 0.13516***
	P-KIZMID-KIR	22.00±0.00	4.25±0.53	2.32±0.14	0.96±0.08	100.00%	0.181±0.075	0.88±0.08	0.57±0.04	-0.56±0.09	
	P-KIZMID-KRK	27.75±0.16	6.50±0.65	2.80±0.23	1.22±0.09	100.00%	0.220±0.074	0.81±0.07	0.63±0.04	-0.30±0.08	
	P-KIZDOWN-COR	14.00±0.00	3.5±0.19	2.14±0.14	0.85±0.07	100.00%	0.194±0.087	0.88±0.10	0.53±0.05	-0.68±0.09	
	P-IHR-NEV	23.63±0.32	5.63±0.60	2.84±0.37	1.16±0.13	100.00%	0.162±0.057	0.85±0.10	0.61±0.06	-0.38±0.12	
	Mean	21.48±0.73	4.70±0.28	2.53±0.11	1.04±0.05	100.00%	0.191±0.079	0.87±0.04	0.59±0.02	-0.48±0.05	

N: sample size, Na: mean allele number, Ne: effective number of alleles, I: Shannon's Information Index, %P: percentage of polymorphic loci, G-W Index (M): Garza- Williamson index, Ho: observed heterozygosity, uHe: unbiased expected heterozygosity, F: fixation index, FST: proportion of the diversity in the sample due to allele frequency differences among populations (***:p<0.001,**:p<0.01,*:p<0.05)
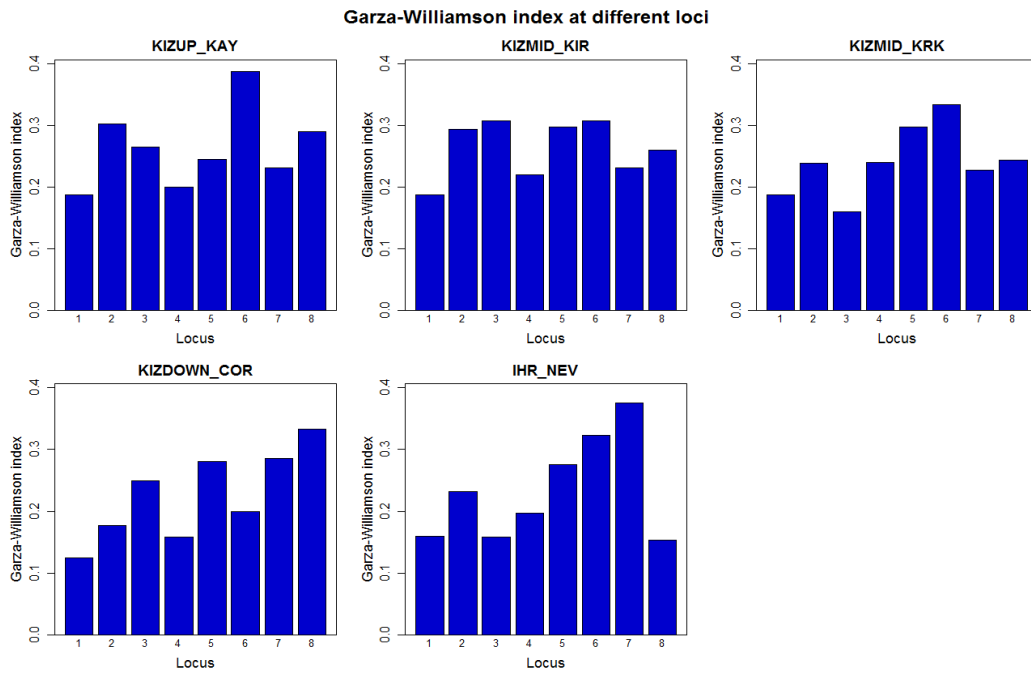
Figure 4.1. Bar graphs, showing that the Garza-Williamson index values across studied loci in each population of *S. alba.*
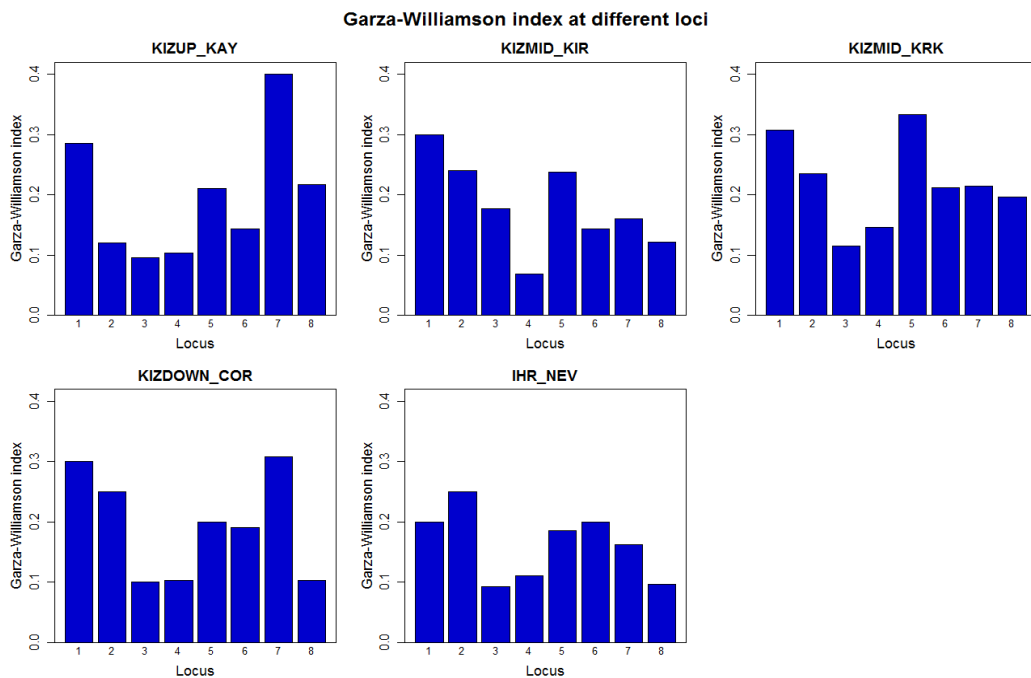
Figure 4.2. Bar graphs, showing that the Garza-Williamson index values across studied loci in each population of *P. nigra.*

4.3.2.1   Analysis of Molecular Variance (AMOVA)

Analysis of Molecular Variance (AMOVA) was performed to estimate the genetic differentiation of populations by Arlequin v3.5.2.2 (Excoffier & Lischer, 2010). Both F_{ST}, the number of different alleles based, and R_{ST}, the sum of squared size differences based, models were carried out for both species data (Table 4.9.)

For *S. alba* populations, F_{ST}-based AMOVA indicated that most of the genetic variation significantly arose from among individuals within populations with a percentage of 94.17% ($F_{ST} = 0.0583$, p = 0.0000). A smaller portion of the total variance was due to the difference between the two rivers which was 4.29% ($F_{CT} = 0.0429$, p = 0.2287). Among populations within rivers had the least contribution to the total variance with 1.54% ($F_{SC} = 0.0161$, p = 0.0000). On the other hand, R_{ST}-based AMOVA results of *S. alba* gave similar outcomes in terms of partitions of total genetic variance yet with slightly different percentages. These were found as 4.43% for among rivers ($F_{CT} = 0.0443$, p = 0.2014), 1.92% among populations within rivers ($F_{SC} = 0.0201$, p = 0.0313), and 93.66% for among individuals within populations ($F_{ST} = 0.0634$, p=0.0000).

F_{ST}-based AMOVA of *P. nigra* populations showed that the largest proportion of the total genetic variation originated from among individuals within populations, as the percentage value of 86.48% ($F_{ST} = 0.13516$, p = 0.0000). Genetic variation among rivers followed it with 12.93% value ($F_{CT} = 0.1293$, p = 0.1955). Then, among populations within rivers had only 0.58% of total variation ($F_{SC} = 0.0067$, p = 0.2444). Likewise, R_{ST}-based AMOVA indicated similar results for *P. nigra*, this time with closer percentages of variation to the F_{ST}-based model. The percentage of total variation due to among individuals within populations was 86.90% ($F_{ST} = 0.1310$, p = 0.0000), due to among rivers was 13.55% ($F_{CT} = 0.1355$, p = 0.1936), and among populations within rivers was -0.45% ($F_{SC} = -0.0052$, p = 0.4721). The slightly negative values like the case of among populations within rivers can be considered as zero due to the working principle of AMOVA estimators with random variables (Schneider et al., 2000).

Table 4.9. Analysis of molecular variance (AMOVA) for *S. alba* and *P. nigra* using eight microsatellite loci between two groups/regions each (The Kızılırmak River and the Melendiz River-Ihlara)

		Source of variation	Sum of squares	Variance components	Percentage of variation	Fixation indices (F_{CT}, F_{SC}, F_{ST})
S. alba populations	F_{ST} Based	Among rivers	15.015	Va = 0.10796	4.29%	F_{CT} = 0.04290
		Among populations within rivers	14.386	Vb = 0.03878	1.54%	F_{SC} = 0.01610***
		Within Populations	751.285	Vc = 2.36999	94.17%	F_{ST} = 0.05831***
		Total	780.686	2.51673		
	R_{ST} Based	Among rivers	3537.986	Va = 24.77625	4.43%	F_{CT} = 0.04427
		Among populations within rivers	3584.197	Vb = 10.72298	1.92%	F_{SC} = 0.02005*
		Within Populations	166160.947	Vc = 524.1703	93.66%	F_{ST} = 0.06343***
		Total	173283.130	559.66625		
P. nigra populations	F_{ST} Based	Among rivers	29.853	Va = 0.34731	12.93%	F_{CT} = 0.12931
		Among populations within rivers	8.910	Vb = 0.01571	0.58%	F_{SC} = 0.00672
		Within Populations	494.777	Vc = 2.32290	86.48%	F_{ST} = 0.13516***
		Total	533.541	2.68592		
	R_{ST} Based	Among rivers	9303.124	Va = 113.64156	13.55%	F_{CT} = 0.13548
		Among populations within rivers	1721.083	Vb = -3.76662	0.00%	F_{SC} = -0.00519
		Within Populations	155256.325	Vc = 728.90293	86.90%	F_{ST} = 0.13099***
		Total	166280.532	838.77787		

(***:p<0.001,**:p<0.01,*:p<0.05)

4.4 Population Genetic Structures

4.4.1 Pairwise F_{ST} Matrices and Principle Coordinate Analyses

Pairwise F_{ST} values and Nm values were calculated for populations of both species and given in Appendix F. Figure 4.5. and Figure 4.6. illustrate the pairwise F_{ST} matrices of *S. alba* and *P. nigra* populations, respectively.

In *S. alba*, all populations significantly differentiated from each other with different F_{ST} values. However, the Ihlara population shows the highest differentiation from all populations of Kızılırmak. Moreover, the downstream population of Kızılırmak (S-KIZDOWN-COR) is the most distant one among the populations of the river.
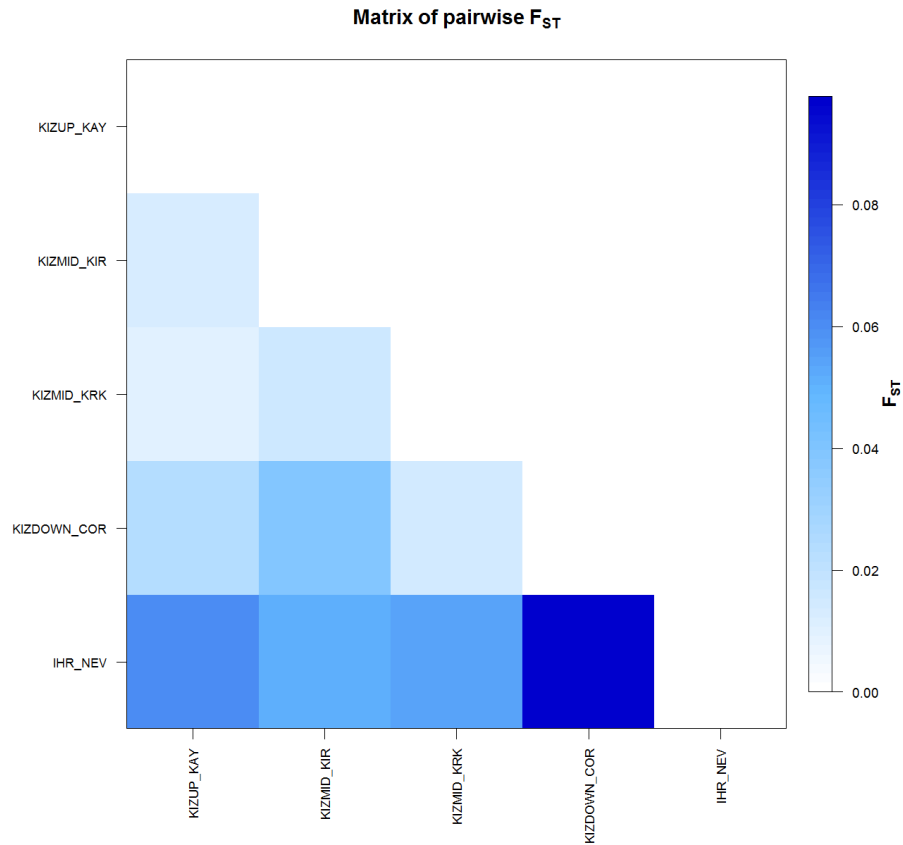
Figure 4.3. Heat map representation of pairwise F_{ST} matrix of *S. alba* populations

The Ihlara population of *P. nigra* is significantly differentiated from all populations of Kızılırmak. Kızılırmak populations do not show a considerable differentiation from each other, and differentiation of them is difficult to interpret with pairwise F_{ST} values.
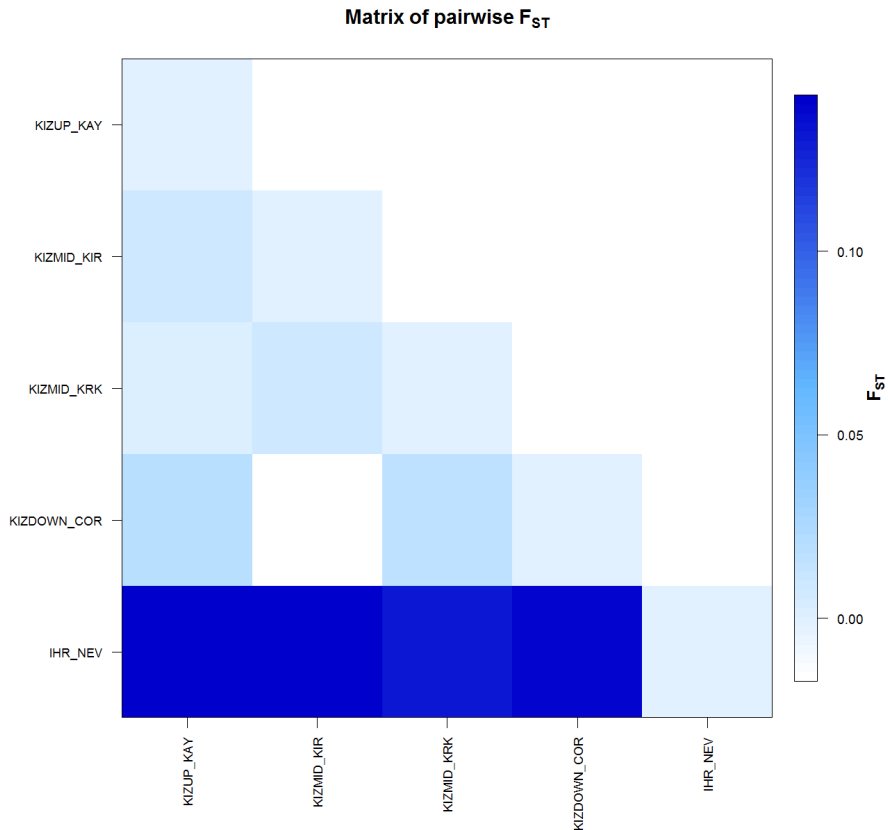
Figure 4.4. Heat map representation of pairwise F_{ST} matrix of *P. nigra* populations

Principal Coordinates Analyses (PCoA) were carried out based on the pairwise F_{ST} matrices of *S. alba* and *P. nigra* by using GenAlEx 6.503 (Peakall & Smouse, 2012). PCoA gives an idea about genetic variation distribution structure over the geographical location of samples (McVean, 2009). PCoA results were given in Figure 4.5. for *S. alba* populations and in Figure 4.6. for *P. nigra* populations. For both species, two rivers were distinctly separated from each other by the first principle coordinate which explains 60.09% of the variation for *S. alba* populations

and 70.85% for *P. nigra* populations. Also, the second principle coordinate explains the 24.51% and 19.46% of the variations respectively for *S. alba* and *P. nigra* populations. On the other hand, populations of Kızılırmak were not separated from each other as clear as the Ihlara population for both species as well.

Principal Coordinates (PCoA) of *S. alba* Populations

◆ S_KIZDOWN-COR

◆ S_IHR-NEV

◆ S_KIZMID-KRK

◆ S_KIZUP-KAY

◆ S_KIZMID-KIR

Coord. 2 (24.51%)

Coord. 1 (60.09%)
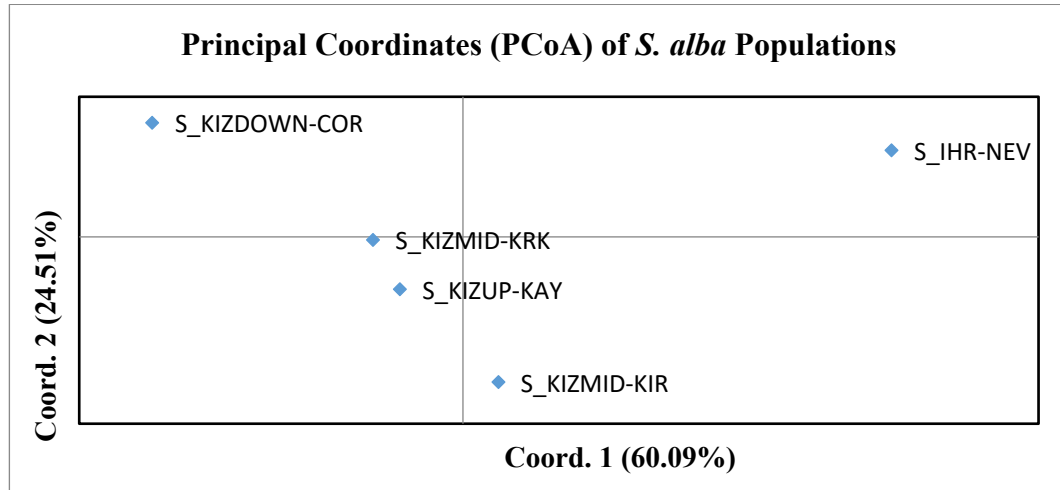
Figure 4.5. Principal Coordinate Analysis (PCoA) of *S. alba* populations based on pairwise F_{ST} values

Principal Coordinates (PCoA) of *P. nigra* Populations

◆ P_KIZUP-KAY
◆ P_KIZMID-KRK

◆ P_IHR-NEV

◆ P_KIZMID-KIR

◆ P_KIZDOWN-COR

Coord. 2 (19.46%)

Coord. 1 (70.85%)
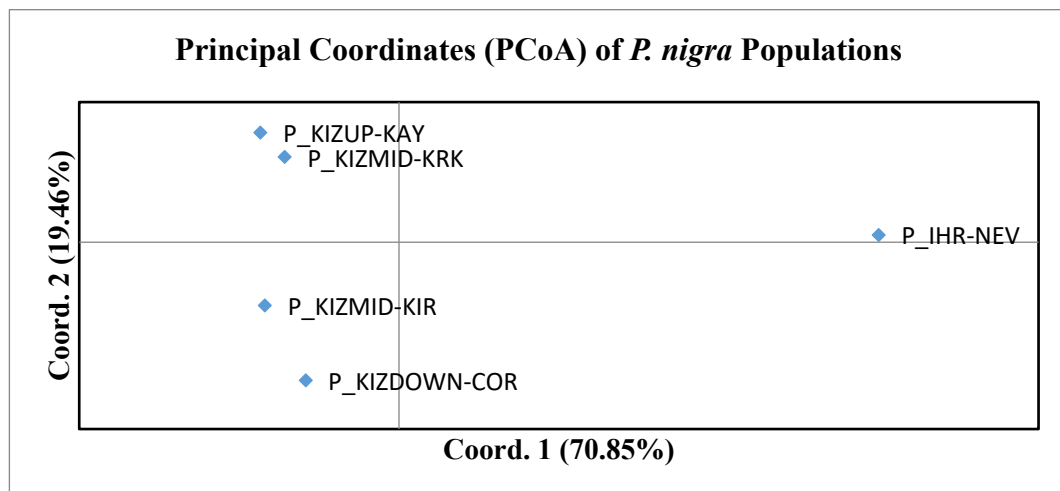
Figure 4.6. Principal Coordinate Analysis (PCoA) of *P. nigra* populations based on pairwise F_{ST} values

4.4.2 Phenetic Relationship Among Populations of Two Species

To reveal the phenetic relationships among the populations of *S. alba* and *P. nigra*, two different phenograms were constructed based on the UPGMA method and provided in Figure 4.7. and Figure 4.8. respectively. Although Ihlara populations of both species formed a distinct clade than all of the Kızılırmak populations, Kızılırmak populations of two species showed different subcluster structures among themselves.
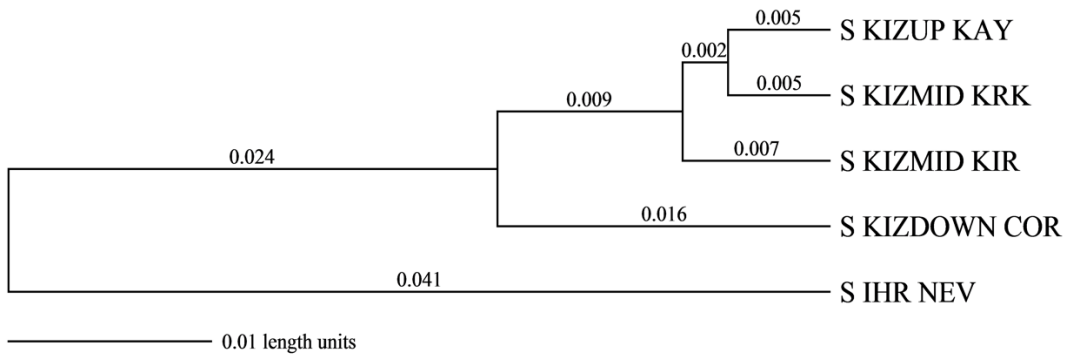
Figure 4.7. UPGMA tree of *S. alba* populations
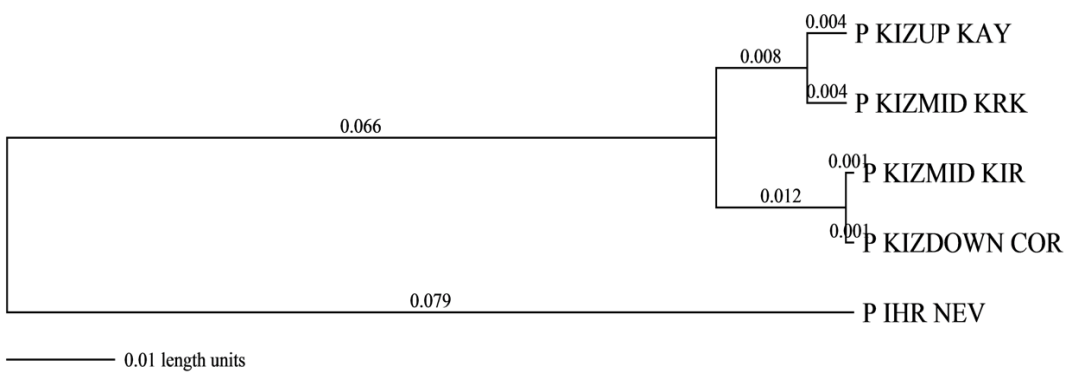
Figure 4.8. UPGMA tree of *P. nigra* populations

40

4.4.3   Genetic Clustering Patterns

Genetic structures and clustering patterns of studied *S. alba* and *P. nigra* populations were analyzed via STRUCTURE v2.3.4 (Pritchard et al., 2000) with an iterative Bayesian algorithm to assign individuals to different numbers of clusters with and without prior information about the populations of the individuals. These analyses were carried out for all populations together. Since the analyses with and without prior information resulted in similar ways, results of the data without prior information were provided only in the study.

S. alba populations were assessed primarily, and among 1 to 5 tested K values, K=2 were detected as the true number of clusters since it has the highest ΔK and lowest standard deviation values according to Evanno method analysis (Evanno et al., 2005) (Table 4.10. and Figure 4.9.). It can be interpreted that these two clusters point out the structural difference of two rivers, Kızılırmak and Melendiz. The genetic difference between the two rivers was presented in Figure 4.10. which showed most of the genotypes of the Ihlara population were assigned to the same cluster as a comparison of Kızılırmak populations.

Table 4.10. Evanno table, showing the estimated ΔK values for clustering of *S. alba* populations

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-4022,19	0,4202	NA	NA	NA
2*	10	-4015,44	5,4985	6,75	90,53	16,464415
3	10	-4099,22	137,2525	-83,78	196,02	1,42817
4	10	-3986,98	30,63	112,24	234,96	7,670909
5	10	-4109,7	39,4502	-122,72	NA	NA
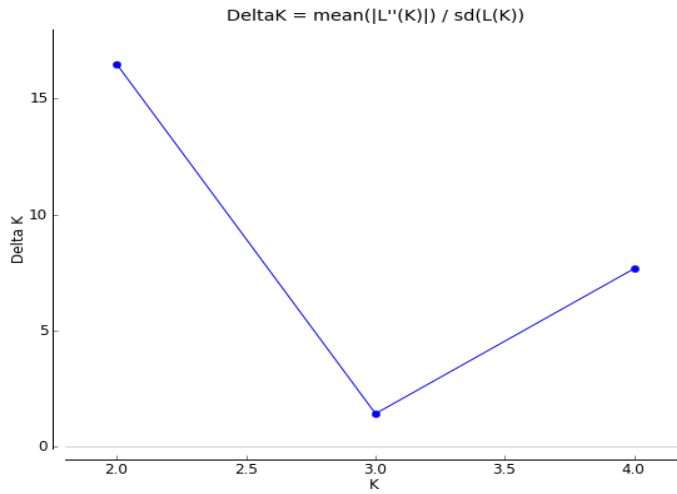
Figure 4.9. Line graph of ΔK values of *S. alba* populations
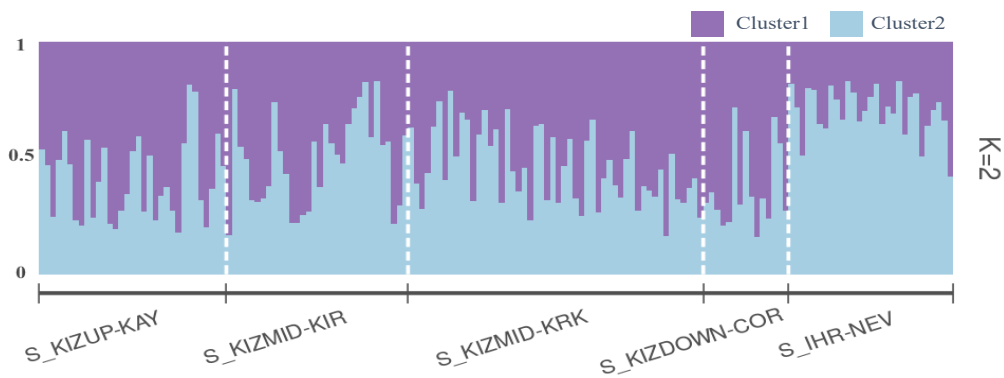
Figure 4.10. Clustering patterns of *S. alba* populations based on genetic structure analysis results (K=2)

Subsequently, the same analyses were done for *P. nigra* starting from the STRUCTURE (Pritchard et al., 2000) analysis. As a result, Table 4.11. and Figure 4.11. were constructed by using the Evanno method (Evanno et al., 2005). K=4 was selected as the true cluster number value among 1 to 5 K values by evaluating the highest ΔK and the lowest standard deviation. Clustering patterns graph in Figure 4.12. shows clear discrimination between the Ihlara and Kızılırmak river populations compared to the *S. alba*. Members of the Ihlara population were mostly assigned to the Cluster4 while the majority of the individuals of Kızılırmak populations were distributed to the first three clusters.

42

Table 4.11. Evanno table, showing the estimated ΔK values for clustering of *P. nigra* populations

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-22.908.200	0.3360	NA	NA	NA
2	10	-20.620.300	87.952	228.790.000	41.300.000	4.695.739
3	10	-18.745.400	59.781	187.490.000	95.870.000	16.036.746
4*	10	-17.829.200	0.8149	91.620.000	173.360.000	212.747.693
5	10	-18.646.600	243.695	-81.740.000	NA	NA
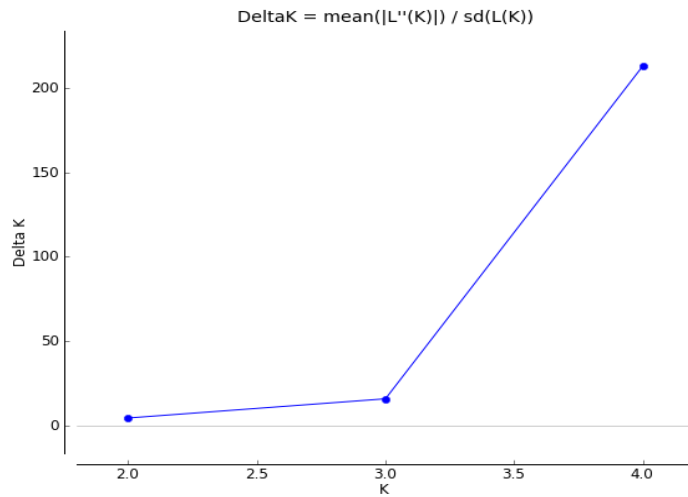
Figure 4.11. Line graph of ΔK values of *P. nigra* populations
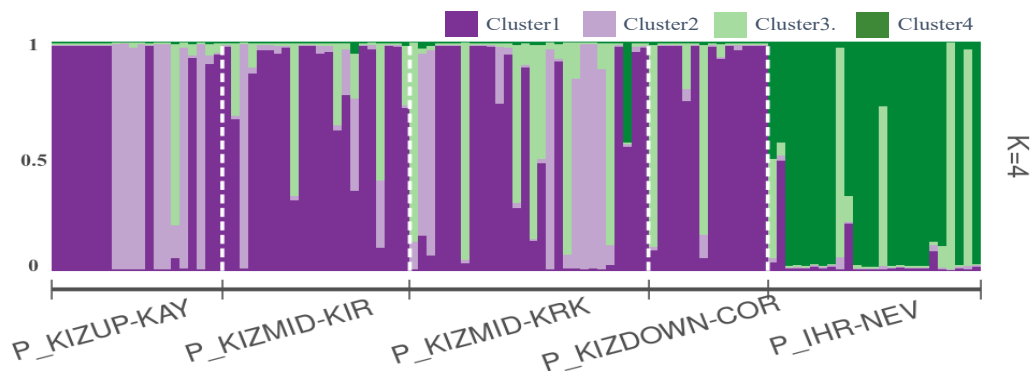
Figure 4.12. Clustering patterns of *P. nigra* populations based on genetic structure analysis results (K=4)

CHAPTER 5

DISCUSSION

In this thesis, the effects of habitat fragmentation in terms of genetic structures of *Salix alba* and *Populus nigra* populations have been elucidated for the first time by comparing the genetic data, obtained using eight microsatellite markers, from a highly fragmented and protected river systems of Turkey, namely Kızılırmak and Melendiz. This information is important to understand the level of change in the genetic diversities and population structures of these two species based on the human-related activities that alter the environment basically and develop strategies to attenuate the negative impact of those activities on the species. Moreover, this study may play a role in guiding the establishment of further conservation and breeding programs.

5.1   Genetic Diversities of the Microsatellite Loci

All of the studied eight SSR loci were polymorphic for all populations of both species. Polymorphism information content (PIC) was determined to reveal the informativeness of the studied loci and Shannon's information index (I) value is used to evaluate the efficiency of the SSR markers for the identification of species diversity (Gill & Joanes, 1979). For *S. alba*, all loci, except WPMS18 and WPMS16, were highly informative. WPMS18 and WPMS16 loci are not suitable to be used without any informative locus in population genetic studies of *S. alba*. For *P. nigra*, all loci were highly informative except for the WPMS04 which was reasonably informative.

45

Allelic richness (Ar) is another estimation for the genetic diversity of a population that takes account of the long-term evolutionary potential of the population (Allendorf, 1986; Petit et al., 1998). The highest Ar value belongs to the WPMS04 locus, and WPMS16 has the lowest Ar value, which is the only value lower than 3.0 for *S. alba*. The PMGC2163 locus had the highest Ar, and the lowest Ar value belonged to WPMS04 loci for *P. nigra*. Furthermore, heterozygosity levels of loci gave similar information as allelic richness. Therefore, the WPMS04 locus could be considered as an effective and informative locus for *S. alba* populations. Likewise, the PMGC2163 locus was the most efficient and revealing one among the studied loci for *P. nigra*. These findings are parallel to the previous study of Ciftci et al. (2019) in terms of heterozygosity of the loci of *P. nigra* populations. However, the WPMS04 locus has not been studied for *S. alba* previously.

In *S. alba* populations, four loci showed an excess of heterozygosity with negative F_{IS} values, and three of them significantly deviated from the Hardy-Weinberg Equilibrium. The remaining four loci had positive F_{IS} values indicating the heterozygote deficiency, and three of them significantly deviated from the Hardy-Weinberg Equilibrium. In *P. nigra* populations, only one locus, WPMS04, had a positive F_{IS} value, and it is the only one that is not significantly deviated from the Hardy-Weinberg Equilibrium. Positive fixation index values point out the high level of inbreeding, meaning that the non-random mating among the individuals of a population, with the high probability of being a consequence of human-related activities such as selective breeding or habitat fragmentation. Additionally, deviations from the Hardy-Weinberg Equation might also be caused by non-random mating and reduction in effective population size as a result of human-related actions (Kansu & Kaya, 2020). Hence, it can be interpreted that habitat fragmentation highly affects these two species in the studied locations.

5.2 Genetic Diversities Among Populations of Two Species

The genetic diversity of a population is a good indicator of a successful population throughout the potential evolutionary events. Polymorphism content, heterozygosity, number of different alleles, and private alleles are used to evaluate genetic diversity (Hughes et al., 2008). In this concept, private alleles have high significance for conservation activities (Funk, Mullins, & Haig, 2007). The number and frequencies of private alleles varied differently for populations of both species. The highest numbers of private alleles belonged to one of the middle populations of Kızılırmak, S-KIZMID-KRK, and Ihlara population, S-IHR-NEV, with 6 private alleles among the *S. alba* populations. The S-KIZMID-KRK population also had a higher observed heterozygosity level than other populations. These findings may be the evidence of this population having a greater effective population size than others, so it should be recognized for new breeding strategies as well as for conservation of the species. However, the *S. alba* downstream population of Kızılırmak, S-KIZDOWN-COR, had only one private allele staggeringly. Likewise, P-IHR-NEV and P-KIZMID-KRK populations of *P. nigra* had the highest number of private alleles which are 16 and 10, respectively. Again, the lowest number of private alleles of *P. nigra* belongs to the P-KIZDOWN-COR population with only two private alleles.

All studied populations of *S. alba* showed a moderate level of genetic diversity according to observed heterozygosity values while all populations of *P. nigra* had the observed heterozygosity with higher magnitudes. For both species, observed heterozygosity (Ho) values of almost all populations were greater than the unbiased expected heterozygosity (uHe) values. The exceptions of that were S-KIZMID-KIR and S-IHR-NEV populations of *S. alba.* However, these values are very close to each other for *S. alba* populations while the differences between uHe and Ho values are greater in *P. nigra* populations. This indicates that there may be greater scale gene flow to *P. nigra* populations than *S. alba*, and also higher adaptability of *S. alba*. These results show high similarities with the findings of the previous studies (Degirmenci et al., 2019; Ciftci et al., 2019).

When genetic diversities of Kızılırmak and Ihlara were compared, no significant difference between these fragmented and protected river systems was discovered. The reason behind that could be the habitat fragmentations and other human practices that affect species in Ihlara valley before 1990, the date it has gone under protection and declared as a Special Environmental Protection Area (Uzun & Somuncu, 2011).

Fixation index (F) values were determined as negative for all populations of both species resulting from the higher observed heterozygosity values than expected. The reasons behind that can be explained by the selection of heterozygotes by human or natural ways, disassortative mating of individuals, and the number of heterozygotes in the initial founder population. The negative F value is an expected outcome of dioecious and highly heterozygous features of the species of the Salicaceae family together with the variation increasing ways of reproduction such as pollination by wind, dispersal of seeds by animals, and easy vegetative propagation (Degirmenci et al., 2019).

Garza-Williamson index (M) values were estimated to reveal whether the studied populations experienced any past bottleneck event (Garza & Williamson, 2001). M values of all populations of both species were lower than the critical value indicating that past bottleneck events had occurred reducing the allelic diversity more rapidly than heterozygosity during the species histories. Also, the detected excess of heterozygosity, characterized with negative F value, in the populations might result from the past bottleneck event of populations (Kikuchi, Suzuki, & Sashimura, 2011). These bottleneck events were most likely derived from habitat fragmentation or destruction by the constructed dams and topography of the river flow. Since habitat fragmentation may lead a genetic drift and genetic isolation in a population, the revealed bottleneck events that occurred in recent history might be caused by habitat fragmentation with a strong probability.

5.3   Genetic Differentiations and Structures of Populations

Detection of differentiation among the populations gives an idea about the evolution of the species. Differentiations among the population are affected by genetic drift positively while gene flow affects it negatively. Since genetic drift is a consequence of habitat fragmentation, genetic differentiation can be evaluated as a sign of habitat fragmentation. Genetic differentiation among the populations of Kızılırmak is low for both species and very close to each other with the F_{ST} values of 0.0156 for *S. alba* and 0.0179 for *P. nigra*. These values were increased to 0.0538 and 0.1352 for *S. alba* and *P. nigra*, respectively with the addition of the Ihlara populations. The low F_{ST} value is an expected result for the species of the Salicaceae family since their pollen and seed can be easily dispersed by wind, water and animals to long distances, and vegetative reproduction is an easy and natural process for these species by broken branches that are floated with the river current (Buchler et al., 2003; Ciftci et al., 2019; Degirmenci et al., 2019; Kansu et al., 2020). The low difference between the F_{ST} values indicates the better protection of the wild nature of *S. alba* populations, and the high difference between the F_{ST} values of *P. nigra* points out more vulnerability of the species to habitat fragmentation and other human-related activities.

Furthermore, analysis of molecular variance (AMOVA) was carried out for both species to reveal the genetic differentiation levels of the two rivers, populations, and individuals together with the origin of the variances. According to the results, most of the total genetic variation was due to the individuals (genotypes) within the populations, and the smallest contribution to the total variation was provided by the differentiation among the populations within rivers of both species. On the other hand, the variation due to the difference between the two rivers was higher than the variation due to the populations within rivers for both species. That indicates the differentiation between the Kızılırmak and Melendiz rivers. If the AMOVA results of *S. alba* and *P. nigra* are compared, the differentiation level of the two rivers was found greater for *P. nigra* while differentiation of populations is lower for the same

species. This implies the gene flow among the populations of *P. nigra* is higher, but it is more vulnerable to human-mediated actions in terms of habitat fragmentation and unregulated cultivation practices.

In the pairwise F_{ST} matrices of both species, the differentiation of the Ihlara populations from the Kızılırmak populations was apparent. All of the *S. alba* populations significantly differ from each other while the Kızılırmak populations of *P. nigra* do not have considerable differentiation from each other, supporting the findings of AMOVA. Moreover, the downstream population of Kızılırmak (S-KIZDOWN-COR) was found to be the most distant one among the populations of the river for *S. alba*. The reason for that might be geographical, like the Black Sea Mountains or constructed dams which create genetic barriers between the downstream population and the rest of the population. This outcome also indicates the effect of habitat fragmentation on the species. Furthermore, these results were also verified and visualized by principal coordinate analysis (PCoA). Similarly, the Ihlara populations of both species were distinctly separated from the Kızılırmak populations. However, separation levels of Kızılırmak populations were not as apparent as the separation of the Ihlara population for both species. Nevertheless, some of the Kızılırmak populations of *P. nigra* were closer to each other as a pattern of pairs. P-KIZUP-KAY was closer to P-KIZMID-KRK, and P-KIZMID-KIR was closer to P-KIZDOWN-COR. On the other hand, S-KIZDOWN-COR was detected as the furthest population among the Kızılırmak populations of *S. alba* while S-KIZMID-KRK and S-KIZUP-KAY were closer to each other.

Differentiation patterns of the populations, that were discovered in pairwise F_{ST} matrices and PCoA, were confirmed by constructing phenograms for both species. Again, Ihlara populations seemed as distinct clades from the Kızılırmak populations for both *S. alba* and *P. nigra*. For *S. alba*, S-KIZDOWN-COR was the most distinctive population among Kızılırmak populations, and S-KIZMID-KIR followed it while S-KIZUP-KAY and S-KIZMID-KRK seemed the closest relatives of each other. For *P. nigra*, populations of Kızılırmak such as P-KIZUP-KAY and P-

KIZMID-KRK created a monophyletic group while P-KIZMID-KIR and P-KIZDOWN-COR formed another monophyletic group.

The analysis determining the genetic structure of 161 *S. alba* individuals revealed that there were two clusters indicating the differentiation between the two rivers. This proves the geographical distance between the populations of the same species affects genetic differentiation. Afterward, genetic structure analyses were performed for 109 individuals of *P. nigra*. Individuals were assigned to four clusters as a result of the analysis, yet discrimination between Ihlara and Kızılırmak populations is very firm. Also, although *P. nigra* populations were found to be assigned into four clusters, two of them showed similarities and could be interpreted as the same cluster. Hence, the true number of clusters for *P. nigra* populations can be evaluated as three. On the other hand, when the structure analyses of the two species are compared, it was clear that genotypes were more equally distributed into the clusters for *S. alba* populations while the genotype distribution patterns into clusters for *P. nigra* populations were more distinctive. Furthermore, the differentiation of the two rivers is not that evident in *S. alba* as in *P. nigra*. Thus, this verifies the previous findings of the study. That is habitat fragmentation and other human-mediated actions have significant impacts on the genetic structure of populations in both species, but *P. nigra* was more heavily influenced by these disturbances than *S. alba*. Furthermore, it can be interpreted that initial diversity in *S. alba* is better conserved and maintained even in fragmented habitats. The reason behind that could be related to the better conservation of wild nature of *S. alba* than *P. nigra*. *P. nigra* has been a more commercially cultivated and transported species by humans in Turkey. Therefore, the more extensive usage areas and human relations of *P. nigra* bring about the genetic diversity loss and genetic pollution in wild populations by selective breeding, clonal forestry, and gene flow from the cultivated trees and hybrids (Vanden Broeck et al., 2004). Hence, the adaptation ability of *S. alba* becomes higher than *P. nigra* to the fragmented habitats.

To sum up, human-mediated actions in Turkey like urbanization, construction of dams and factories, usage of forest lands as agricultural areas led to fragmentations of river systems. These habitat fragmentations result in bottleneck events and genetic diversity decline in populations of the keystone species like *S. alba* and *P. nigra* which are distributed in these river basins. The consequences of the study have significance for understanding the impacts of habitat fragmentation on the genetic and population structures of *S. alba* and *P. nigra* differentially. In the light of the information provided in the current study, further studies focusing on conservation and improvement of biodiversity strategies in Turkey can be conducted, future cultivation, afforestation, and conservation actions can be established for *S. alba* and *P. nigra* in Turkey, agricultural, usage of natural resources and urbanization policies of government can be revised.

CHAPTER 6

CONCLUSION

S. alba and *P. nigra,* which are important tree species of riparian ecosystems, are naturally distributed in almost all river basins of Turkey. The differential impacts of habitat fragmentation on the genetics and population structures of these two species were assessed by using the same microsatellite markers for the comparison of the genotypes which were sampled from different rivers. These rivers were chosen to represent a highly fragmented river ecosystem and a protected river ecosystem from habitat fragmentation. Kızılırmak river is an example of a fragmented river ecosystem, and samples were collected from four different parts of the river composing four different populations. The Melendiz river in Ihlara valley typifies the protected river ecosystem for the study.

The results of the study revealed that with the evaluation of Polymorphism Information Content (PIC), Probability of identity (PI), Allelic richness (Ar), Shannon Index (I), and expected heterozygosity (He), the informative loci were WPMS04, PMGC2709, PMGC2889, PPGC14, PMGC2163 for *S. alba* while the PMGC14, PMGC2163, WPMS18, PMGC2889, WPMS14, PMGC2709 loci were informative for *P. nigra*, and these loci can be preferred for the further genetic diversity assessment studies dealing with these two species.

The highest genetic diversity, in terms of the high number of private alleles, observed in the Ihlara populations together with the Kırıkkale middle population of Kızılırmak for both species. These populations can be considered as the genetic resource of

future breeding and conservation programs of these species. Moreover, natural and human-related gene flow is a very common situation among the populations of two species. The results showed that the gene flow among *S. alba* populations seems to have occurred more in natural ways while the human impact on the gene flow among *P. nigra* populations is considerably higher. This indicates the vulnerability of *P. nigra* to habitat fragmentation and human activities, and conservation actions to prevent the decrease of the genetic diversity of the species are needed.

Genetic structures of the populations of two species are confirmatory of the outcomes from previous studies. Habitat fragmentation and other human-mediated activities change the population structures and reduced the genetic diversity in both species. However, these had a greater impact on *P. nigra* compared to *S. alba*. These two ecologically and economically important species need to be protected from unregulated human activities including habitat destruction and fragmentation for several agricultural and urbanization practices, and inaccurately executed commercial or ornamental cultivations and improper afforestation policies. The information obtained and provided in this study may play an important role in developing and establishing both successful *in situ* and *ex situ* conservation programs and new ways to decrease the negative impacts of habitat fragmentation on these two species together with further studies.

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APPENDICES

A. DETAILED INFORMATION ON THE STUDIED POPULATIONS ACROSS THE KIZILIRMAK AND IHLARA RIVER SYSTEMS

River system	Population code	Province/County	Altitude (m)	River condition	Climate type of the region	Mean annual temperature	Mean annual precipitation (Millimetre)	Length (Km)	Number of dams/HPP
Kızılırmak	KIZUP-KAY	Kayseri/Ürgüp	789-1113	Intermediate, Fragmented	Semi dry-less humid, Semi dry	9.40-12.58°C	443.46-638.10	1,355	15
	KIZMID-KIR	Kırşehir	640-816						
	KIZMID-KRK	Kırıkkale	730-1269						
	KIZDOWN-COR	Çorum	358-424						
Ihlara	IHR-NEV	Ihlara/Nevşehir	1102	Good	Semi dry	11.20°C	398.81	14	-

(Değirmenci et al., 2022, in press)

B. CTAB DNA EXTRACTION PROTOCOL

1. 0.1 gram of leaf tissue was taken from each sample and put in a sterile mortar to ground with 1000 μL 2X CTAB extraction buffer containing CTAB (pH:8.0), Tris-HCl (pH:8.0), EDTA and NaCl.

2. The obtained mixtures were poured into 2 mL Eppendorf tubes and 700 μL CTAB, 200 μL β-mercaptoethanol and 5 μL Proteinase K were added to each tube then, they were incubated at 65°C for 30 minutes.

3. After the incubation completed, the tubes were centrifuged at 15000 rpm and +4°C for 15 minutes.

4. The aqueous phases were collected into new 2 mL Eppendorf tubes and 0.8 V Phenol:Chloroform:Isoamyl alcohol (25:24:1) was added. If the phase is not clear, this step should be repeated.

5. Then, they were centrifuged at 15000 rpm and +4°C for 15 minutes.

6. After centrifugation, the supernatant was transferred to new tubes and 0.8 V Chloroform:Isoamyl alcohol (24:1) was added and inverted a few times gently.

7. The tubes were centrifuged at 15000 rpm +4°C for 15 minutes.

8. Supernatants were taken and 0.7-1 V isopropanol were put onto the mixture. Then, tubes were gently flip-flopped a few times.

9. The samples were incubated at -20°C for 2 hours. After cold incubation, they were centrifuged at 13000 rpm in +4°C for 8 minutes.

10. The pellets were washed with cold 70% ethanol twice.

11. Supernatants were discarded and the tubes with pellet were left inverted on a clean tissue paper for air-dry for 60 minutes.

12. After DNA pellets were dried, they were resuspended in 50-75 μL TE (Tris-HCL (pH:7.0) and EDTA) overnight at +4°C.

C. BUFFERS, CHEMICALS AND EQUIPMENTS

Buffers and solutions for DNA isolation:

2X CTAB: 2 gr CTAB (Cetyl Trimethyl Ammonium Bromide), (SIGMA)

4 ml (pH:8) 0.5 M EDTA, (FLUKA)

10 ml (pH:8) Tris HCL, (SIGMA)

28 ml NaCl is completed with 100 mL distilled water

Phenol, (AMRESCO)**:** Pure phenol

Chloroform isoamyl alcohol, (FLUKA) : (24/1)

Ethanol: 70% in distilled water

B-mercaptoethanol, (SIGMA) : 17,5 ml β-mercaptoethanol is completed with 250 ml with distilled water

TE buffer: 10 mM Tris HCL (pH:7) 10mm ethylene diamine tetra acetic acid disodium salt (EDTA)

Isopropanol, (FLUKA) : Pure Isopropanol, ice cold

Buffers and solutions for PCR:

Sterile water

Taq DNA Polymerase (SIGMA Red *Taq*): 1U/µl

10X PCR buffer including MgCl2 (SIGMA)

dNTPs (SIGMA): 10mM

DNA: 20 ng/µL

Primer Pairs: 10µM

Agarose Gel Electrophoresis Buffers and Gel System:

10X TBE Buffer: 108 gr Trizma Base, (SIGMA), 55 gr Boric Acid, (SIGMA)

Running Buffers: X TBE prepared in distilled water

Ethidium Bromide, (SIGMA): 4 mg/ ml 105

Agarose, (SIGMA): 3 % Agarose Gel

40 ml EDTA, (FLUKA) (0.5 M, pH:8) completed with 1000 ml with distilled water

Low molecular weight DNA Ladder (SIGMA)

Equipments:

Autoclave: Yamato

Centrifuge: Nüve- NF048

Electrophoresis System: Thermo Scientific

Thermocyclers: Eppendorf- Mastercycler

Deep freezer: UĞUR- Freezer

Magnetic Stirrer: Labor Brand – Hotplate L-81

Refrigerator: Siemens

UV Transilluminator: Vilbor Lourmant

Vortex: Nüve- NM110

Water Bath: Memmert

Oven: Dedeoğlu

Micropipettes: Gılson

pHmeter: Hanna Inst.

D. PRIVATE ALLELES BY POPULATIONS OF TWO SPECIES

Private alleles and frequencies of *S. alba* populations

POPULATION	LOCUS	ALLELE	FREQUENCY
S-KIZUP-KAY	PMGC14	224	0,015
S-KIZUP-KAY	WPMS14	219	0,015
S-KIZUP-KAY	PMGC2709	166	0,015
S-KIZUP-KAY	PMGC2889	210	0,015
S-KIZUP-KAY	PMGC2163	186	0,076
S-KIZMID-KIR	PMGC2709	214	0,016
S-KIZMID-KIR	PMGC2889	200	0,016
S-KIZMID-KRK	WPMS16	164	0,010
S-KIZMID-KRK	WPMS04	330	0,029
S-KIZMID-KRK	WPMS04	382	0,048
S-KIZMID-KRK	PMGC2709	198	0,010
S-KIZMID-KRK	PMGC2709	200	0,019
S-KIZMID-KRK	PMGC2889	178	0,010
S-KIZDOWN-COR	PMGC2889	222	0,033
S-IHR-NEV	WPMS16	152	0,017
S-IHR-NEV	WPMS16	176	0,034
S-IHR-NEV	PMGC14	233	0,017
S-IHR-NEV	WPMS18	217	0,086
S-IHR-NEV	WPMS18	223	0,052
S-IHR-NEV	PMGC2163	226	0.017

Private alleles and frequencies of *P. nigra* populations

POPULATION	LOCUS	ALLELE	FREQUENCY
P-KIZUP-KAY	WPMS04	264	0,050
P-KIZUP-KAY	PMGC2709	208	0,025
P-KIZMID-KIR	PMGC14	213	0,023
P-KIZMID-KIR	WPMS14	216	0,023
P-KIZMID-KIR	WPMS14	225	0,023
P-KIZMID-KRK	WPMS16	156	0,018
P-KIZMID-KRK	PMGC14	225	0,036
P-KIZMID-KRK	WPMS04	254	0,018
P-KIZMID-KRK	WPMS04	256	0,018
P-KIZMID-KRK	WPMS04	286	0,018
P-KIZMID-KRK	PMGC2709	200	0,019
P-KIZMID-KRK	PMGC2889	180	0,018
P-KIZMID-KRK	WPMS18	252	0,018
P-KIZMID-KRK	PMGC2163	206	0,019
P-KIZMID-KRK	PMGC2163	220	0,019
P-KIZDOWN-COR	WPMS14	249	0,036
P-KIZDOWN-COR	PMGC2163	252	0,036
P-IHR-NEV	WPMS16	135	0,022
P-IHR-NEV	WPMS16	159	0,304
P-IHR-NEV	PMGC14	189	0,021
P-IHR-NEV	PMGC14	216	0,063
P-IHR-NEV	PMGC2709	202	0,023
P-IHR-NEV	PMGC2709	216	0,023
P-IHR-NEV	PMGC2889	216	0,040
P-IHR-NEV	PMGC2889	218	0,040
P-IHR-NEV	WPMS18	246	0,458
P-IHR-NEV	WPMS18	261	0,042
P-IHR-NEV	PMGC2163	186	0,042
P-IHR-NEV	PMGC2163	192	0,125
P-IHR-NEV	PMGC2163	198	0,229
P-IHR-NEV	PMGC2163	210	0,042
P-IHR-NEV	PMGC2163	242	0,229
P-IHR-NEV	PMGC2163	268	0,042

E. ALLELIC PATTERNS ACROSS THE POPULATIONS

Allelic patterns across *S. alba* populations
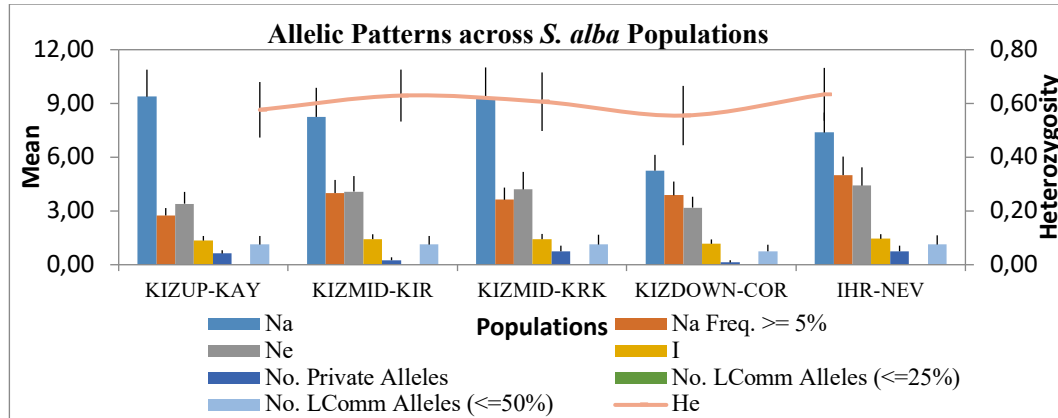
(Na: Number of Different Alleles, Na (Freq≥5%): Number of Different Alleles with a Frequency≥5%, Ne: Number of Effective Alleles, I: Shannon's Information Index, No. Private Alleles: Mean Number of Alleles Unique to a Single Population, No. LComm Alleles (≤25%) Number of Locally Common Alleles (Freq.≥5%) Found in 25% or Fewer Populations, No. LComm Alleles (≤50%) Number of Locally Common Alleles (Freq.≥5%) Found in 50% or Fewer Populations, He: expected heterozygosity)

Allelic patterns across *P. nigra* populations
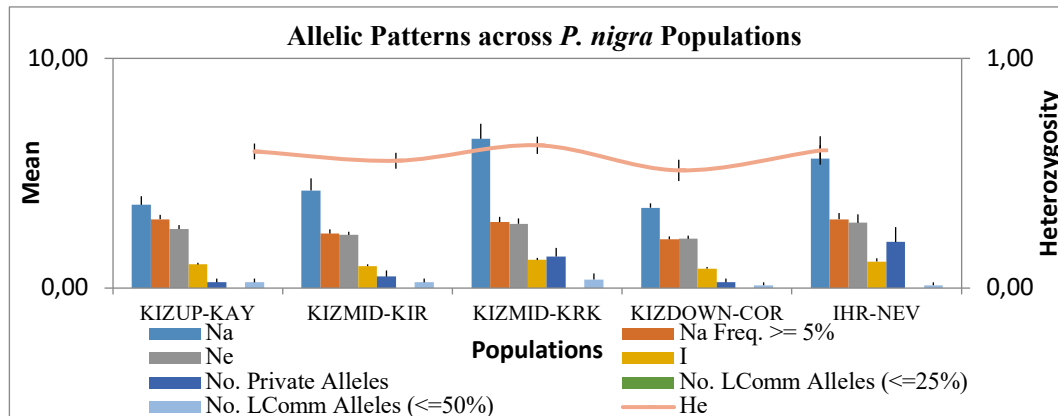
(Na: Number of Different Alleles, Na (Freq≥5%): Number of Different Alleles with a Frequency≥5%, Ne: Number of Effective Alleles, I: Shannon's Information Index, No. Private Alleles: Mean Number of Alleles Unique to a Single Population, No. LComm Alleles (≤25%) Number of Locally Common Alleles (Freq.≥5%) Found in 25% or Fewer Populations, No. LComm Alleles (≤50%) Number of Locally Common Alleles (Freq.≥5%) Found in 50% or Fewer Populations, He: expected heterozygosity)

F. PAIRWISE F_{ST} MATRICES OF BOTH SPECIES

Pairwise F_{ST} values (below the diagonal) and Nm (Number of migrants) values (above the diagonal) of *S. alba* populations

	KIZUP-KAY	KIZMID-KIR	KIZMID-KRK	KIZDOWN-COR	IHR-NEV
S-KIZUP-KAY		19.962	26.410	10.296	3.871
S-KIZMID-KIR	0.012*		16.070	6.181	4.603
S-KIZMID-KRK	0.009***	0.015**		17.807	4.375
S-KIZDOWN-COR	0.024**	0.039***	0.014**		2.309
S-IHR-NEV	0.061***	0.052***	0.054***	0.098***	

(***:p<0.001,**:p<0.01,*:p<0.05)

Pairwise F_{ST} values (below the diagonal) and Nm (Number of migrants) values (above the diagonal) of *P. nigra* populations

	KIZUP-KAY	KIZMID-KIR	KIZMID-KRK	KIZDOWN-COR	IHR-NEV
P-KIZUP-KAY		15.737	24.528	9.717	2.849
P-KIZMID-KIR	0.009		18.449	32.554	2.991
P-KIZMID-KRK	0.001	0.008		11.052	3.116
P-KIZDOWN-COR	0.018	-0.017	0.017		3.173
P-IHR-NEV	0.142***	0.141***	0.132***	0.139***	

(***:p<0,001,**:p<0,01,*:p<0,05)

G. POPULATION GENETICS STATISTICS

Number of different alleles (Na):

Detected by direct count.

Effective number of alleles (Ne):

Ne: The estimation of the number of equally frequent alleles in an ideal population

He: Expected heterozygosity

$$Ne = \frac{1}{1 - He}$$

Number of private alleles:

The number of alleles unique to a single population in the data set.

Shannon's Information Index (I):

I: Calculated on a single-locus basis in equivalence of the Shannon-Weaver Index of ecology

ln: Natural logarithm

p_i: The frequency of the ith allele of the particular locus of a population

$$I = \sum pi \ln pi$$

Observed Heterozygosity (Ho):

N: Population size

$$Ho = \frac{Number\ of\ Heterozygotes}{N}$$

Expected Heterozygosity (He):

p_i: The frequency of the ith allele of the particular locus of a population

$$He = 1 - \sum pi^2$$

Fixation Index (F):

F: Fixation index, inbreeding coefficient

He: Expected heterozygosity

Ho: Observed heterozygosity

$$F = \frac{He - Ho}{He}$$

Wright's F-Statistics:

F_{IS}: The inbreeding coefficient within individuals relative to the populations

$\overline{He}$: The mean of expected heterozygosity averaged across populations

$\overline{Ho}$: The mean of observed heterozygosity averaged across populations

$$FIS = \frac{\overline{He} - \overline{Ho}}{\overline{\overline{He}}}$$

F_{ST}: The inbreeding coefficient within populations relative to the total population

H_T: The expected heterozygosity

$\overline{He}$: The mean of expected heterozygosity averaged across populations

$$FST = \frac{H_T - \overline{He}}{H_T}$$

F_{IT}: The inbreeding coefficient within individual relative to the total population

H_T: The expected heterozygosity

$\overline{Ho}$: The mean of observed heterozygosity averaged across populations

$$FST = \frac{H_T - \overline{Ho}}{H_T}$$

Relation equation of F-statistics:

$$(1 - FIT) = (1 - FIS).(1 - FST)$$

Number of migrants (Nm):

F_{ST}: The inbreeding coefficient within populations relative to the total population

$$Nm = \frac{\left[\left(\frac{1}{FST}\right) - 1\right]}{4}$$

Probability of Identity (PI)

p_i: The frequency of the ith allele of the particular locus of a population.

$$PI = 2\left(\sum pi^2\right)^2 - \sum pi^4$$

Polymorphic Information Content (PIC)

p_i: The frequency of the ith allele of the particular locus of a population.

$$PIC = 1 - \sum (pi^2)$$

Percentage of Polymorphic Loci (%P)

p_i: The frequency of the ith allele of the particular locus of a population.

N: The number of populations

$$P = \sum \frac{pi}{N}$$

Garza-Williamson Index (G-W)

k: Number of alleles at a particular locus

R: Allelic range

$$GW = \frac{k}{R+1}$$

H. INPUT FILE FORMATS OF THE SOFTWARES

Arlequin File Format (.arp)

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  Title = "Populus.txt"
  NbSamples = 5
  DataType = MICROSAT
  GenotypicData = 1
  LocusSeparator = WHITESPACE
  MissingData = "?"
  GameticPhase = 0
  RecessiveData = 0


[Data]
  [[Samples]]
    SampleName = "KIZUP-KAY"
    SampleSize = 20
    SampleData = {
        P_KUP-1   1       144 195 210 274 190 192 225 224
                          150 201 243 274 194 212 231 240
        P_KUP-2   1       144 195 210 274 190 192 225 224
                          150 201 243 274 194 212 231 240
        P_KUP-3   1       144 195 210 274 190 192 225 224
                          150 201 243 274 194 212 231 240
        P_KUP-4   1       144 195 210 274 190 192 225 224
                          150 201 243 274 194 212 231 240
        P_KUP-5   1       144 195 210 274 190 192 225 224
                          150 201 243 274 194 212 231 240
        P_KUP-6   1       144 195 210 274 190 192 225 224
                          150 201 243 274 194 212 231 240
        P_KUP-7   1       144 195 210 274 190 192 225 224
                          150 201 243 274 194 212 231 240
        P_KUP-8   1       144 201 231 246 190 200 231 224
                          150 219 243 274 204 212 234 228
        P_KUP-9   1       144 201 231 246 190 200 231 224
                          150 219 231 274 204 212 234 228
        P_KUP-10      1       144 201 231 246 190 200 231 224
                              150 219 261 274 204 212 234 228
        P_KUP-11      1       144 201 231 246 190 200 231 224
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        P_KUP-12      1       144 195 210 274 190 192 225 224
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        P_KUP-13      1       144 201 231 246 190 200 231 224
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        P_KUP-15      1       150 201 210 264 190 192 228 224
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        P_KUP-16      1       144 201 231 246 190 200 231 224
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        P_KUP-17      1       144 195 210 274 194 192 225 224
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        P_KUP-18      1       144 201 231 246 190 200 231 224
                              150 219 243 274 204 212 234 228
        P_KUP-19      1       144 195 210 274 190 192 225 224
                              150 201 243 274 194 212 231 246
        P_KUP-20      1       144 195 210 274 190 192 225 224
                              150 201 237 274 194 212 231 240
    }
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Cervus File Format (.csv)

Population	ID	WPMS16A	WPMS16B	PMGC14A	PMGC14B	WPMS14A	WPMS14B	WPMS04A	WPMS04B	PMGC2709A	PMGC2709B	PMGC2889A	PMGC2889B	WPMS18A	WPMS18B	PMGC2163A	PMGC2163B
Pop1	S_KUP-1	158	158	197	197	204	216	264	276	194	212	182	192	220	220	198	198
Pop1	S_KUP-2	158	158	200	218	198	204	268	280	166	194	202	206	220	220	198	198
Pop1	S_KUP-3	158	158	194	194	198	219	268	292	194	212	194	202	220	220	198	204
Pop1	S_KUP-4	158	173	200	200	204	222	256	290	210	212	192	206	220	226	194	198
Pop1	S_KUP-5	158	158	194	200	198	216	268	274	204	208	186	202	220	220	198	198
Pop1	S_KUP-6	158	158	194	197	192	204	274	284	210	210	192	194	226	232	186	198
Pop1	S_KUP-7	158	158	194	200	198	204	252	276	208	210	194	202	220	220	186	198
Pop1	S_KUP-8	158	158	194	200	198	204	266	276	194	212	194	194	220	220	186	198
Pop1	S_KUP-9	158	158	194	206	198	204	252	274	194	208	192	202	220	226	198	208
Pop1	S_KUP-10	158	158	194	200	198	204	274	282	212	212	182	192	220	220	198	198
Pop1	S_KUP-11	158	158	185	194	198	204	266	272	208	208	194	202	220	226	198	198
Pop1	S_KUP-12	158	158	194	203	198	204	256	280	194	210	194	202	226	226	198	200
Pop1	S_KUP-13	158	158	200	200	198	204	272	290	194	208	194	202	220	226	198	198
Pop1	S_KUP-14	158	158	194	203	198	204	274	290	194	212	194	202	220	220	198	198
Pop1	S_KUP-15	158	158	215	218	198	204	264	284	194	210	194	194	220	220	198	204
Pop1	S_KUP-16	158	158	188	194	198	204	294	304	194	194	194	202	220	220	198	198
Pop1	S_KUP-17	158	158	194	194	204	210	282	286	210	212	192	210	220	220	186	198
Pop1	S_KUP-18	158	158	194	194	189	198	276	288	210	212	192	212	220	220	186	198
Pop1	S_KUP-19	158	158	200	200	198	204	274	274	212	212	182	192	220	220	198	198
Pop1	S_KUP-20	158	158	197	200	189	204	282	294	210	218	192	202	220	220	198	198
Pop1	S_KUP-21	158	158	197	206	198	204	282	290	210	212	192	202	220	220	198	198
Pop1	S_KUP-22	158	158	197	197	204	204	256	266	194	212	192	204	220	226	198	198
Pop1	S_KUP-23	158	158	197	197	204	204	274	296	212	216	192	208	220	220	204	212
Pop1	S_KUP-24	158	158	194	197	198	204	276	284	194	210	194	202	220	220	198	198
Pop1	S_KUP-25	158	158	197	200	198	204	276	294	210	212	192	202	220	220	198	198
Pop1	S_KUP-26	158	158	182	197	198	204	258	274	202	216	204	204	220	220	198	204
Pop1	S_KUP-27	158	158	197	197	204	204	258	274	180	184	198	206	220	220	198	216
Pop1	S_KUP-28	158	158	194	197	201	210	268	274	190	210	184	192	220	220	194	210
Pop1	S_KUP-29	158	158	200	224	198	204	272	272	212	216	192	202	220	220	198	212
Pop1	S_KUP-30	158	158	197	200	198	204	276	282	190	210	192	192	220	220	198	212
Pop1	S_KUP-31	158	167	197	203	198	204	262	282	192	212	194	202	220	220	198	212
Pop1	S_KUP-32	158	158	197	212	198	204	256	280	192	212	206	206	220	220	198	198
Pop1	S_KUP-33	158	158	191	191	198	204	278	288	190	210	184	192	220	220	198	198
Pop2	S_KM-KI-34	158	158	197	200	198	204	260	268	208	210	192	202	220	220	198	212
Pop2	S_KM-KI-35	158	158	185	194	204	204	278	282	190	212	184	192	226	226	198	200
Pop2	S_KM-KI-36	158	158	197	200	198	204	286	304	190	210	182	192	220	226	198	212
Pop2	S_KM-KI-37	158	158	197	203	198	204	256	272	172	172	190	192	220	220	198	212
Pop2	S_KM-KI-38	158	158	194	200	204	204	280	286	190	210	184	192	220	220	198	198
Pop2	S_KM-KI-39	158	158	194	200	198	204	272	288	172	194	190	194	220	220	198	198
Pop2	S_KM-KI-40	158	158	194	215	198	198	274	288	194	208	194	202	220	220	198	198
Pop2	S_KM-KI-41	158	158	197	197	198	204	256	272	194	194	190	194	220	220	198	204
Pop2	S_KM-KI-42	158	158	191	215	204	210	262	278	194	210	194	202	226	226	198	200
Pop2	S_KM-KI-43	158	158	194	203	198	204	268	282	194	210	194	212	220	220	198	198
Pop2	S_KM-KI-44	158	158	197	197	198	204	270	292	208	210	192	202	220	232	198	198

81

FSTAT File Format (.dat)

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WPMS14
WPMS04
PMGC2709
PMGC2889
WPMS18
PMGC2163
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GDA File Format (.nex)

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    format  separator=/ missing=?;
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        2 PMGC14,
        3 WPMS14,
        4 WPMS04,
        5 PMGC2709,
        6 PMGC2889,
        7 WPMS18,
        8 PMGC2163;
    matrix
        KIZUP_KAY:
            S_KUP-1   158/158 197/197 204/216 264/276 194/212 182/192 220/220 198/198
            S_KUP-2   158/158 200/218 198/204 268/280 166/194 202/206 220/220 198/198
            S_KUP-3   158/158 194/194 198/219 268/292 194/212 194/202 220/220 198/204
            S_KUP-4   158/173 200/200 204/222 256/290 210/212 192/206 220/226 194/198
            S_KUP-5   158/158 194/200 198/216 268/274 204/208 186/202 220/220 198/198
            S_KUP-6   158/158 194/197 192/204 274/284 210/210 192/194 226/232 186/198
            S_KUP-7   158/158 194/200 198/204 252/276 208/210 194/202 220/220 186/198
            S_KUP-8   158/158 194/200 198/204 266/276 194/212 194/194 220/220 186/198
            S_KUP-9   158/158 194/206 198/204 252/274 194/208 192/202 220/226 198/208
            S_KUP-10  158/158 194/200 198/204 274/282 212/212 182/192 220/220 198/198
            S_KUP-11  158/158 185/194 198/204 266/272 208/208 194/202 220/226 198/198
            S_KUP-12  158/158 194/203 198/204 256/280 194/210 194/202 226/226 198/200
            S_KUP-13  158/158 200/200 198/204 272/290 194/208 194/202 220/226 198/198
            S_KUP-14  158/158 194/203 198/204 274/290 194/212 194/202 220/220 198/198
            S_KUP-15  158/158 215/218 198/204 264/284 194/210 194/194 220/220 198/204
            S_KUP-16  158/158 188/194 198/204 294/304 194/194 194/202 220/220 198/198
            S_KUP-17  158/158 194/194 204/210 282/286 210/212 192/210 220/220 186/198
            S_KUP-18  158/158 194/194 189/198 276/288 210/212 192/212 220/220 186/198
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            S_KUP-20  158/158 197/200 189/204 282/294 210/218 192/202 220/220 198/198
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            S_KM-KI-34  158/158 197/200 198/204 260/268 208/210 192/202 220/220 198/212
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            S_KM-KI-42  158/158 191/215 204/210 262/278 194/210 194/202 226/226 198/200
            S_KM-KI-43  158/158 194/203 198/204 268/282 194/210 194/212 220/220 198/198
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            S_KM-KI-47  158/158 200/218 198/198 278/292 212/212 192/202 220/220 198/224
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            S_KM-KI-50  158/158 197/197 198/204 282/292 194/194 194/202 226/232 198/206
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GenAlEx File Format (.xlsx)

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	Salix				S_KIZUP-KA	S_KIZMID-K	S_KIZMID-K	S_KIZDOWN	S_IHR-NEV									
		Pop	WPMS16		PMGC14		WPMS14		WPMS04		PMGC2709		PMGC2889		WPMS18		PMGC2163	
1	S_KIZUP-KAY	158	158	197	197	204	216	264	276	194	212	182	192	220	220	198	198	
2	S_KIZUP-KAY	158	158	200	218	198	204	268	280	166	194	202	206	220	220	198	198	
3	S_KIZUP-KAY	158	158	194	194	198	219	268	292	194	212	194	202	220	220	198	204	
4	S_KIZUP-KAY	158	173	200	200	204	222	256	290	210	212	192	206	220	226	194	198	
5	S_KIZUP-KAY	158	158	194	200	198	216	268	274	204	208	186	202	220	220	198	198	
6	S_KIZUP-KAY	158	158	194	197	192	204	274	284	210	210	192	194	226	232	186	198	
7	S_KIZUP-KAY	158	158	194	200	198	204	252	276	208	210	194	202	220	220	186	198	
8	S_KIZUP-KAY	158	158	194	200	198	204	266	276	194	212	194	194	220	220	186	198	
9	S_KIZUP-KAY	158	158	194	206	198	204	252	274	194	208	192	202	220	226	198	208	
10	S_KIZUP-KAY	158	158	194	200	198	204	274	282	212	212	182	192	220	220	198	198	
11	S_KIZUP-KAY	158	158	185	194	198	204	266	272	208	208	194	202	220	226	198	198	
12	S_KIZUP-KAY	158	158	194	203	198	204	256	280	194	210	194	202	226	226	198	200	
13	S_KIZUP-KAY	158	158	200	200	198	204	272	290	194	208	194	202	220	226	198	198	
14	S_KIZUP-KAY	158	158	194	203	198	204	274	290	194	212	194	202	220	220	198	198	
15	S_KIZUP-KAY	158	158	215	218	198	204	264	284	194	210	194	194	220	220	198	204	
16	S_KIZUP-KAY	158	158	188	194	198	204	294	304	194	194	194	202	220	220	198	198	
17	S_KIZUP-KAY	158	158	194	194	204	210	282	286	210	212	192	210	220	220	186	198	
18	S_KIZUP-KAY	158	158	194	194	189	198	276	288	210	212	192	212	220	220	186	198	
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23	S_KIZUP-KAY	158	158	197	197	204	204	274	296	212	216	192	208	220	220	204	212	
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31	S_KIZUP-KAY	158	167	197	203	198	204	262	282	192	212	194	202	220	220	198	212	
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33	S_KIZUP-KAY	158	158	191	191	198	204	278	288	190	210	184	192	220	220	198	198	
34	S_KIZMID-KIR	158	158	197	200	198	204	260	268	208	210	192	202	220	220	198	212	
35	S_KIZMID-KIR	158	158	185	194	204	204	278	282	190	212	184	192	226	226	198	200	
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Genepop File Format (.gen)

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PMGC2889
WPMS18
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Microchecker File Format (.txt)

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