DETERMINATION OF GENETIC DIVERSITY IN *SALIX CAPREA* POPULATIONS FROM THE CORUH RIVER WATERSHED

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DETERMINATION OF GENETIC DIVERSITY IN *SALIX CAPREA* POPULATIONS FROM THE CORUH RIVER WATERSHED

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ABSTRACT

DETERMINATION OF GENETIC DIVERSITY IN *SALIX CAPREA* POPULATIONS FROM THE CORUH RIVER WATERSHED

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The genus willow (*Salicaceae*) has around 500 species worldwide, 27 species naturally distributed in Turkey. Goat willow (*Salix caprea*) is an ecologically important, cold tolerant pioneer species which spreads in Europe and western and central Asia, in the Mediterranean Sea, central Anatolia and Black Sea regions in Turkey. It is also naturally distributed through Coruh river banks. Hydroelectric dam plant dams are being built in these regions which endanger this species. Some of the *Salix caprea* populations would be under water in near future. Beside this, hydroelectric plants may change local climate which would affect *Salix caprea* negatively by increasing average annual temperature. Moreover, there have been genetic diversity studies in Europe about *Salix caprea* species by using SSR markers, but there has been no study related with genetic diversity assessment of this species in Turkey yet. Therefore, it is important to determine the genetic diversity of *Salix caprea* populations in the eastern Black Sea Region.

To characterize genetic diversity of *Salix caprea* species, 180 genotypes were analyzed by using 10 microsatellite markers. The number of observed alleles ranged from 3.500 to 5.600 and average mean value was 4.770. The observed heterozygosity

ranged from 0.082 to 1.000 and average mean value was 0.503 while expected heterozygosity ranged from 0.119 to 0.827 and average mean value was 0.538. % 7.5 genetic variation was between populations and % 92.5 genetic variations were within populations. When *Salix caprea* populations were evaluated according to their genetic relations, two separate groups were formed; one group included populations in the Trabzon region and the other group included populations in the Artvin region.

In the current study, magnitude and pattern of genetic diversity of *Salix caprea* were determined successfully. Populations had moderate level of genetic diversity. Construction of dams in these regions might endanger genetic resources of *Salix caprea* in near future.

Key Words: *Salix caprea*, microsatellites, genetic diversity, allele data, population structure

ÖZ

ÇORUH HAVZASINDA DOĞAL YAYILIŞ GÖSTEREN SALİX CAPREA POPULASYONLARININ GENETİK ÇEŞİTLİLİĞİNİN BELİRLENMESİ

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Söğüt cinsi (*Salicaceae*) dünyada 500 civarı türe sahiptir, 27 türü Türkiye'de doğal olarak yayılmıştır. Keçi söğüdü (*Salix caprea*) Avrupada ve batı ve orta Asyada, Türkiye'de Akdeniz, orta Anadolu, ve Karadeniz bölgelerinde yayılmış olan ekolojik olarak önemli, soğuğa toleranslı öncü bir türdür. Çoruh nehir kıyısı boyunca da doğal olarak yayılmıştır. Bu bölgede bu türü tehlikeye sokan hidroelektrik santrali barajları inşa edilmektedir. Gelecekte bazı keçi söğüdü populasyonları su altında kalacak. Buna ek olarak, yerel klima değişimi keçi söğüdünü negatif olarak etkileyebilir. Ayrıca, SSR markörleri kullanarak *Salix caprea* türü üzerine Avrupada genetik çeşitlilik çalışmaları vardır fakat Türkiye'de henüz bu türün genetik çeşitliliğinin belirlenmesiyle ilgili bir çalışma yoktur. Bu sebeple Doğu Karadeniz bölgesindeki *Salix caprea* populasyonlarının genetik çeşitliliğini belirlemek önemlidir.

Salix caprea türünü karakterize etmek için, 180 genotip 10 mikrosatellit belirteç kullanarak analiz edildi. Gözlenen alel sayısı 4.770 ortalamayla 3.500 ile 5.600 aralığındaydı, gözlenen heterozigotluk 0.503 ortalamayla 0.082 ile 1.000 aralığındaydı buna rağmen beklenen heterozigotluk 0.119 ile 0.827 aralığındaydı. % %7.5 genetik varyasyon populasyonlar arasında ve % 92.5 genetik varyasyon populasyonlar içindeydi. Populasyonlar genetik ilşkilerine göre değerlendirildiğinde,

iki ayrı grup oluştu; Trabzon bölgesi populasyonlarını içeren grup ve Artvin bölgesi populasyonlarını içeren diğer grup.

Bu çalışmada, *Salix caprea* türünün genetik çeşitliliğinin yapısı ve büyüklüğü belirlendi. Populasyonlar orta seviye genetik çeşitliliğe sahiptiler. Bu bölgede barajların yapılması *Salix caprea* türünün genetic kaynaklarının gelecekte tehlikeye girmesine sebep olabilir.

Anahtar Kelimeler: *Salix caprea*, mikrosatellitler, genetik çeşitlilik, alel verisi, populasyon yapısı

To My Parents

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LIST OF ABBREVIATIONS

CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribose Triphosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
GDA	Genetic Data Analysis
MCMC	Markov Chain Monte Carlo
PCR	Polymerase Chain Reaction
SSR	Simple Sequence Repeat
TBE	Tris Boric Acid EDTA (Ethylene diamine tetra acetic acid disodium salt)
IUCN	International Union for Conservation of Nature

CHAPTER 1

INTRODUCTION

1.1. Genus Salix

Salicaceae family has two main genera; *Salix* and *Populus* (Argus, 1997). *Salix* genus includes high numbers of species varieties among them. There are 350 species according to Skvortsov (1968), 526 species according to Fang (1987), 450 species according to Argus (1997) and 450 species according to Lauron-Moreau (2015). It is accepted that *Salix* L. genus includes 450–520 species (Wu *et al.*, 2015). Willow genus grows on moist soils, cold and temperate regions of northern hemisphere (Argus, 1997) and 275 species in China (Liu *et al.*, 1999), 125 species in Russia, around 100 species in North America and 65 species in Europe (Argus, 1997). There are 23 species according to Davis (1965), 28 species according to Arıhan and Güvenç (2009), 27 species according to Terzioğlu (2014) in Turkey. Four of them are endemic; *S. trabzonica*, *S. purpurea*, *S. rizeensis* ve *S. anatolica.*

Salix genus is deciduous shrub or tree. It has alternate leaves and grows rapidly (Yaltırık 1993). Willow is a dioecious plant and the flowers of willow occur in catkins, early in the spring before leaves and produce high amounts of pollen and very small clumped seeds. The pollination occurs mostly by insects, occasionally with wind. The seeds can be dispersed by wind and water (Skvortsov and Edmundson, 1982).

The Willow genus can reproduce asexually and sexually. Willows can root very readily from cuttings (Laird, 1999). It is also very cross-compatible, various hybrids can occur naturally as well as in cultivation (Mosseler, 1990). It has the same chromosome number with poplars (X = 19, 2n = 2X = 38)(Dai *et al.*, 2014).

Willow is mainly used in medicine and manufacturing. Salicin is metabolized into salicylic acid (a precursor of aspirin) in the human body and the species has some secondary metobolites which have the potential to treat various diseases (Ahmed *et. al.*, 2011). Willow wood is used in manufacture of boxes, chairs, cricket bats, basketry and other furniture toys, hedge, pulp and paper industry, wands and whistles (Ericson, 1984; Ager *et. al*, 1986; Siren *et. al.*, 1979). Moreover, willow also can be used as a source of sustainable and renewable biomass for the bioenergy, biofuel and bioproduct industries (Hanley and Karp, 2013).

1.1.1. *Salix caprea*

1.1.1.1 Biology and ecology

Salix caprea (goat willow) is a deciduous plant which can be shrub form with 2-3 m or tree form with 6-8 m height (Yücel *et. al.*, 1995). The trunk of the plant is grey and fissured, the branches are thick and brownish color for the first year. The leaves are matt green and glabrous as upper surface; grey-green and hairy as bottom surface and they are 5-12 cm long and 3-5.5 cm wide, broader than other wilow species. The buds are spiral with red-brownish color and 3x2 cm size. The leaves and the buds align alternately (Figure 1.1)(Newsholme, 1992).

Salix caprea is a dioecious plant, the male and female catkins are on different plants. The flowers are soft silky and silvery catkin form. Catkins appears before leaves. Male catkin is yellow at polen, oblong ovoid 2-3.5 cm, stamens 2 cm, filaments glabrous, anthers 1.2 mm while female catkin is pale green, elongating to 3-7 cm in fruit, capsule 6-8 mm, ovoid conical, pedicel 4-6 cm (Figure 1.2)(Davis, 1965). Female catkins include seeds which have cottony hairs which assist them as wind dispersal. The seeds are about 0,2 mm size with the fine hairs for dispersal (Meikle, 1984).

A B

Figure 1.1 A. The goat willow shrubs in Camburnu Natural Park, Trabzon, Turkey, B. The leaf of goat willow

A B

C

Figure 1.2 A. Goat willow male catkins with pollen, B. Goat willow female catkins, C. Goat willow female catkins with seeds (retrieved from species of UK, 05.11.2016)

Salix caprea can grow moist soil in sunny places. It generally prefers river edges for growth, but it can also grow humid forest area and field edges. The species is known as sensitive to acidic soil. The growth rate is high. It can propagate vegetatively. The seeds germinate easily, but they can lose their germination ability quickly. Therefore, in landscape gardening cutting propagation is preferred (Avcı, 1999).

1.1.1.2 Taxonomy

Goat willow (*Salix caprea*) is an angiosperm of the genus *Salix* belonging to the family *Salicaceae.* The detailed classification was given in Table 1.1 (Argus, 1997). It has two subspecies; *Salix caprea* subsp. *sphacelata* spread in north England and Scotland region, *Salix caprea* subsp. *caprea* spread in central, south eastern and northern Europe (Meikle, 1984).

Table 1.1 The clasification of *Salix caprea*

Kingdom	Plantae (Plants)
Subkingdom	Tracheobionta (Vascular plants)
Superdivision	Spermatophyta (Seed plants)
Division	Magnoliophyta (Flowering plants)
Class	Magnoliopsida (Dicotyledons)
Subclass	Dilleniidae
Order	Salicales
Family	Salicaceae (Willow family)
Genus	*Salix* L. (willow)
Species	*Salix caprea* L. (goat willow)

1.1.1.3 Distribution

Goat willow can grow sunny, moist or dry, acidic soils next to river banks or borders of forest (Avcı, 1999). Considering this growth habits, it spreads from Spain to England through Japan, from Turkey to Fennoscandia and Siberia distributing up to 70° north latitude (Enescu *et al.*, 2016) . Beside this, the species has been introduced in eastern North America (Argus, 2010).

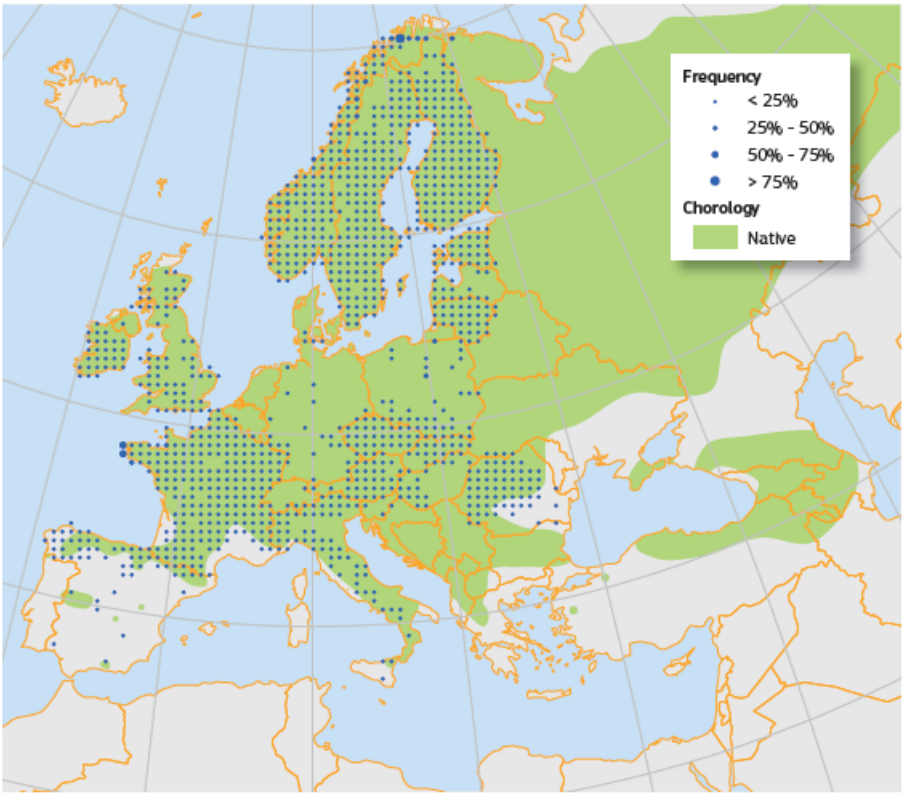

Figure 1.3 The green area shows natural distribution of goat willow in the Europe. (retrieved from Enescu *et al.*, 2016 on 7.11.2016).

1.1.1.4 Importance and usage

Goat willow (*Salix caprea*) grows fast, shoots forths with high amount, can reproduce vegetatively (Ericson, 1984). These features of goat willow make it an important tree in terms of ecological, economic and medicinal interests.

Goat willow has ecological importance because it is pioneer tree, having high dispersal rate under favor of seeds with a coma of cottony hairs, for forestation of open areas or deforested areas due to forest fire or human activity like mining

(Arıhan 2003). Moreover, it can be used to restore damaged ecosystems. The goat willow, cooperation with Ectomycorrhizal (EM) fungi, can be used in recovery of polluted sites based on its high adaptive potential and on its capacity to absorb and accumulate heavy metals (Varga *et al.*, 2009).

The goat willow has economic value, it can be used as biomass for bioenergy and biofuel industries (Hanley and Karp, 2013). Moreover, it is used to make basketry, willow flutes and hedge. Beside this, it has large and soft leaves used as an animal feed (Ericson, 1984; Ager *et. al.*, 1986; Siren *et al.*, 1979).

The goat willow is an important medicinal plant due to having anti-oxidants such as luteolin, dihydrokaempferol and quercetin; having antifungal properties by obtaining some flavonoids; and having salicin in the lieves, used in aspirin (Ahmed *et. al.*, 2011). It has also anti-inflammatory property that is used as antiseptic, eye tonic, analgesic and cardiotonic in Indian System of Medicine (Chopra et al., 1996; Bhattacharjee, 1998; Hollman, 1991; Tunon et al., 1995).

Apart from being ecological, economical and medicinal usage, goat willow is also used as soil retainer where places erosion risky sheer drops owing to ability to root easily and spread vastly. Beside this, it is also used in landscape gardening by virtue of aesthetic appearance of drooping branch with large leaves (Küçükgöksel, 2010).

1.1.1.5 Threats

Genetic diversity of species is important for survival and adaptability of a species. Having a diverse gene pool means that there is high variety in the traits of individuals of a population. When enviroment changes, having high genetic diversity populations' adaptability and survivability increase (Pullin, 2002). On the other hand, loss of genetic diversity, caused by inbreeding depression, contributes to extinction risk in most wild populations of naturally outbreeding species (Frankham, 2005).

Salix caprea spreads through Black Sea Region, especially along with the Coruh river banks. Hydroelectric power plants are being built in these regions. According to 4628 electricity market law, 16 Hydroelectric dams and 157 river type Hydroelectric

power plants will be built onto the Coruh river (Taşkın *et al.,* 2014). Therefore, *Salix caprea* might be in danger as losing genetic diversity or in extinct in those regions.

1.1.1.6 Genetic studies

Genetic diversity is vital for species to adapt variable environmental conditions. Therefore, assessing the genetic diversity of populations is an important task. A microsatellite, simple sequence repeat (SSR), a set of short repeated DNA sequences at a particular locus in nuclear genome and it varies in number in different individuals. Having high level of polymorphism and being co-dominant make them suitable tool for exploring genetic diversity (Freeland *et al.*, 2011).

There are many studies on genus *Salix* by using microsatellites. Nuclear and chloroplast microsatellites were used to detect population genetic structure and reproduction dynamics in *Salix reinii* (Lian *et al.,* 2003), conservation and restoration of *S. lanata* and *S. lapponum* in the UK (*Stamati* et al., 2007), comparing the genetic diversity and structure of *S. eriocephala* and *S. purpurea* L. populations (Lin *et al., 2009*), population genetic structure of endangered *Salix daphnoides* in the Czech Republic (Sochor *et al., 2013*). On the other hand, there are a few study about genotype structure of *S. caprea* by SSR markers. These are; the chloroplast DNA variation in 24 European populations of *Salix caprea* L. (Palme *et al.,* 2003), differentiation of metallicolous and non-metallicolous *Salix caprea* populations (Puschenreiter *et al.*, 2010), and genetic diversity and levels of gene flow in Irish populations of *Salix caprea* L. inferred from chloroplast and nuclear SSR markers in europe about *S. caprea* (Perdereau *et al.,* 2014).

1.1.1.7 *Salix caprea* populations in Turkey

Salix caprea grows Black Sea (North Anatolian mountains), Mediteranean Sea, and central Anatolia regions in Turkey (Avcı, 1999). According to Flora of Turkey and East Aegean Islands (Davis, 1965), it is found in Kırklareli: Istranca Dağı east of Velika, İstanbul: Karaburun, Bursa: Uludağ, Bolu: Aladağ, Zonguldak: Kel Tepe above Karabük, Gümüşhane: Kösedağ, Ordu: Gürgentepe, Artvin: Hopa, Kars: Sarıkamış, Balıkesir: Kaz Dağı, Erzincan: Refahiye, Erzurum: Sonamer Su, Bitlis:

above Sez Köyü, Peşmen, Kars Küçük Ağrı Dağı (2300 m altitude). According to Küçükgöksel (2010), it also grows in Bartın: Yılanlımeşe, Yılansu, Çömlekkıran, Dinencetepe,Yıldırımçukuru,Yaylacıktepe, Ankara: Kızılcahamam: Kızılcaören-Gökdere, Eylek Kayası, and Aluçdağı. *Salix caprea* is also known to grow in Hatilla valey (Emianağaoğlu, 1996), Yusufeli (Aydın Regulator HES Project Final Environmental Impact Assessment Report, 2014), Borcka (Emianağaoğlu *et al.*, 2007) in Artvin.

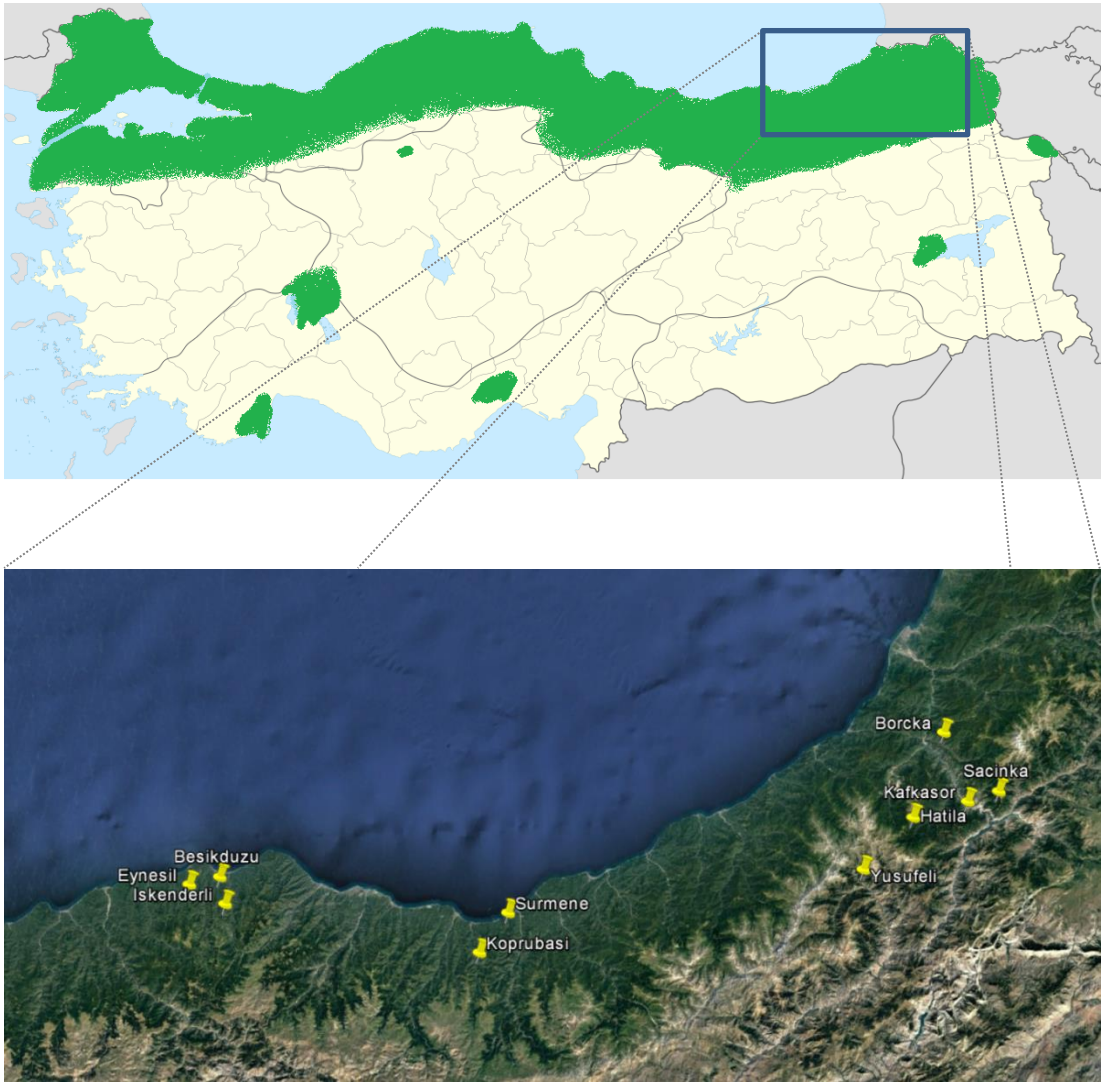

Figure 1.4 Natural distribution of *Salix caprea* in Turkey and locations of samples

1.1.1.8 Justification of the study

Main natural distribution zone of *Salix caprea* species is in the Black Sea region in Turkey. Alpine tourism and especially building dams in this region mihgt have a catastrophic effect on the genetic resources of species. Moreover, although many studies present genetic information of *Salix caprea*, none has been done in Turkey. By this study, genetic diversity pattern of *Salix caprea* populations will be characterised and whether their genetic resources are endangered will be learned. Moreover, if needed, genetic resources of *Salix caprea* would conserved and managed efficiently by this study.

CHAPTER 2

OBJECTIVES OF THE STUDY

The general objective of this study was to asses magnitude and pattern of genetic diversity of *Salix caprea* populations from eastern Black Sea Region by using nuclear SSR markers.

The specific objectives of the study were;

- To test existing nuclear SSR markers for their ability to detect and describe genetic diversity and differentiation of populations in *S. caprea* in eastern Black Sea Region

- To determine the magnitude of genetic diversity of *S. caprea* populations in eastern Black Sea Region

- To describe pattern of genetic diversity of *S. caprea* populations.

CHAPTER 3

MATERIALS AND METODS

3.1. Plant materials

A hundred and eighty eight *Salix caprea* (goat willow) samples were collected from the eastern Black Sea Region of Turkey. Populations' numbers, sampling coordinates were given in Table 3.1. The geographic informaiton on studied populations were given in detail in Appendix A.

Table 3.1 Populations' picked locations, codes and number of samples

Location	Code of population	Number of samples in population	Lattitude of population	Longtitude of population	Altitude of population (average)(metre)
Eynesil (Giresun)	EYN	17	41.04562 N 40.98463 N	39.14716 E 39.11444 E	131-911 (679)
Kafkasör (Artvin/Çoruh)	KAF	8	41.17306 N 41.16722 N	41.81250 E 41.80528 E	861-985 (917)
Sürmene (Trabzon)	SU	18	40.92421 N 40.90783 N	40.22490 E 40.21444 E	66-352 (202)
Beşikdüzü (Trabzon)	BES	27	41.04498 N 41.00217 N	39.29028 E 39.22724 E	35-328 (207)
Köprübaşı (Trabzon)	KPB	15	40.80633 N 40.8016 N	40.14268 E 40.12343 E	322-457 (374)
Borçka (Artvin/Çoruh)	BOR	23	41.37300 N 41.32505 N	41.83321 E 41.73330 E	357-1391 (962)
Saçinka (Artvin/Çoruh)	SAC	15	41.20182 N 41.18721 N	41.91773 E 41.89313 E	1187-1721 (1412)
Yusufeli (Artvin/Çoruh)	YUS	16	41.02504 N 40.88095 N	41.43821 E 41.34472 E	1124-1506 (1348)
İskenderli (Trabzon)	ISK	23	40.93923 N 40.92958 N	39.25489 E 39.24319 E	726-1005 (850)
Hattila (Artvin/Çoruh)	HAT	26	41.14600 N 41.11942 N	41.68290 E 41.60625 E	840-1853 (1405)

3.2. DNA isolation and quantification

Leaves of *Salix caprea* genotypes were collected from September to October of 2015, and stored silica gels until drying. Then, leaves were crushed by using liquid nitrogen and stored in -80 ^{o}C till DNA extraction.

A modified CTAB (Cetyl Trimethyl Ammonium Bromide) method was used to isolate nuclear DNA from leaves of 180 goat willow trees. The details of the method is given in Appendix B (Doyle and Doyle, 1987). After DNA was isolated, DNA pellets were dried by using laminar flow and dissolved in 75 mL TE buffer.

The Nanodrop Spectrophotometer was used to measure isolated DNA concentrations (Thermo Scientific, Wilmington, USA). Isolated DNA quality was obtained by checking the 260:280 OD ratios and by controling whether it suits as a template in the PCR (Polymerase Chain Reaction) with selected SSR primers.

3.3. Microsatallite primers

Ten microsatallite DNA primer pairs were used to investigate magnitude and structure of genetic diversity in *Salix caprea* populations. The primers determined as Karp_W293, SB196, SB24 from Barker *et al.* (2003), WPMS12 from Van Der Schoot *et al*. (2000), gSlMCT052, gSIMCT24 from Stamati et *al.* (2003), WPMS18, WPMS15, WPMS14 from Smulders *et al*. (2001), and Sare04 from Lian *et al.* (2001) were selected in this study. They were synthesized by SACEM Hayat Teknolojileri A.Ş. with different fluorescent dyes which are Fam, Hex, and Tamra. The purpose of using multiple dyes was that it is necessary to recognize PCR products during fragment analysis when multiple dyes were used in order to apply multiple reading. After PCR, three different fluorescent dyed primers were mixed and sent to fragment analysis and later evaluated together. The details of primer sequences and dyes were provided in Appendix C.

3.4. PCR (polymerase chain reaction) optimization

To apply PCR, water, 10X PCR buffer, $MgCl_2$ (25 mM), dNTP mixture (10 mM), 10 microsatellite primer pairs (10 mM) and isolated DNA' (10 ng) were used. For each primer set different PCR conditions were tried. The optimized PCR conditions for 10 SSR primer pairs set were given in Table 3.2.

Table 3.2 PCR optimization conditions for SSR primer-pairs used in this study

SSR locus	H_2O (µl)*	10x PCR buffer (µl)*	$MgCl_2$ (25mM) (µl)*	dNTP mixture (10mM) (µl)*	Primer (10mM) (µl)*	*Taq* DNA polimerase (5u/µl) (µl)*	DNA (10ng /µl) (µl)*	Hot start application	Total Volume (µl)*
WPMS12	9	4	4	O.5	1+1	0.5	5	Hot start	25
WPMS18	9.5	4	4	1	1+1	0.5	4	Hot start	
WPMS14	16.1	2	3	0.3	0.2+0.2	0.2	3	Hot start	
WPMS 15	13.3	3	3	0.5	0.4+0.4	0.4	4	Hot start	
SARE04	13.9	3	3	0.5	0.6+0.6	0.4	3	Hot sart	
SB24	14	3	3	0.6	0.6+0.6	0.2	3	Hot start	
gSlMCT052	15.1	2	3	0.5	0.4+0.4	0.1	3,5	Hot start	
SB196	11.7	3	4	0.4	0.3+0.3	0.3	5	Hot start	
gSIMCT24	8.4	4	3	1	0.5+0.5	0.3	5	Hot start	22,7
Karp_W293	8.4	4	3	1	0.5+0.5	0.3	5	Hot start	

***** represents the amount of concentration added to the PCR reaction.

To amplify the microsatellite regions, PCR cycle conditions were optimized according to Van Der Schoot *et al*. (2000), Smulders *et al*. (2001), Barker *et al.* (2003), and Lian *et al.* (2001), a depending on the primers PCR regions were amplified by using thermocycler (Eppendorf-Mastercycler, Eppendorf, Canada).

Hot start procedure was applied in order to avoid a non-specific amplification of DNA by inactivating the *Taq* polymerase at lower temperatures. For hot start, *Taq* polymerase was added right before second denaturation step. The PCR amplification conditions for each primer were given in Table 3.3.

Table 3.3 PCR amplification conditions for SSR primer-pairs used in this study

Primer Name	Step	Temperature	Time	Number of Cycles	Description
SB24, SB196 (Barker *et al*,. 2003), gSlMCT052 (Stamati *et al.* 2003)	1	94°C	3 min.	1	Denaturation
	2	94°C	1 min.	35	Denaturation
		SB24-57 °C	40 sec.		Annealing
		GS- 60.2 °C			
		S196- 54 °C			
		72°C	1 min.		Extension
	3	95 °C	10 min.	1	Final Extension
SARE04 (Lian *et al.*, 2001)	1	94 °C	3 min.	1	Denaturation
	2	94 °C	1 min.	30	Denaturation
		53 °C	30 sec.		Annealing
		72 °C	1 sec.		Extension
	3	94 °C	5 min.	1	Final Extension
KARP_W293 (Barker et al., 2003), gSIMCT24 (Stamati et al., 2003)	1	94 °C	3 min.	1	Denaturation
	2	94 °C	40 min.	30	Denaturation
		57 °C gSIMCT24	45 sec.		Annealing
		55 °C Karp_W293			
		72 °C	1 min.		Extension
	3	94°C	20 min.	1	Final Extension
WPMS18 (Smulders et al., 2001)	1	94°C	3 min.	1	Denaturation
	2	94°C	30 sec.	35	Denaturation
		53.5°C	45 min.		Annealing
		72°C	45 sec.		Extension
	3	94°C	10 min.	1	Final Extension

Table 3.3 (Continued)

WPMS15, WPMS14 (Smulders et al., 2001)	1	94°C	3 sec.	1	Denaturation
	2	94 °C	40 sec.	35	Denaturation
		W15-54.6 °C	1 min.		Annealing
		W14-54.6 °C			
		72°C			
		72°C	1 min.		Extension
	3	94°C	10 min.	1	Final Extension
WPMS12 (Van Der Schoot *et al*., 2000)	1	94°C	3 min.	1	Denaturation
	2	94°C	40 sec.	35	Denaturation
		51°C	45 sec.		Annealing
		72°C	1 min.		Extension
	3	72°C	10 min.	1	Final Extension

To check the PCR products, gel electrophoresis was applied after DNA amplifications were completed. A 3% agarose gel, stained with 5 µl (g/mL) ethidium bromide was prepared and put in electrophoresis tank filled with 1X TBE (0,4 M Tris Boric acid EDTA) buffer. 5 µl PCR amplification product was loaded in the gel. Low molecular weight DNA ladder (SIGMA) was also loaded to detect the size of bands. The gel was run at 130V for 20 minutes and visualized under UV light. The gels with desired bands were recorded by gel imaging system (Vilbor Lourmat, France).

3.5. Determining the sizes of alleles

PCR amplification products of the individuals were sent for fragment analysis to a company (BM Laboratory, Çankaya, Ankara). The allele determination after fragment analysis was made by Peak Scanner Software v1.0 (Thermo Fisher Scientific, 2015). For each primer pairs, each alleles were analysed and recorded. A

matrix was created in excel which relates genotypes with their loci for each primer pairs (Appendix D).

3.6. Analysis of data

To check sampled genotypes whether they were duplicated due to clonal propagation, Genclone2 software were used. (Arnaud-Haond *et al.*, 2007).

Null allele estimations of *Salix caprea* populations were made by using Genepop 4.2 (Raymound and Rousset, 1995).

3.6.1. Genetic variation estimation

The standart parameters of genetic variability such as number of different alleles (Na), number of effective alleles (Ne), proportion of polymorphic loci (P), observed heterozygosity (Ho), expected heterozygosity (He) were computed. Moreover, F indices, which are also used to define the distribution of genetic variation were calculated. These parameters were obtained by using GenAlEx software (GenAlEx 6.5, Peakal and Smause, 2012).

Proportion of polymorphic loci

It shows the percentage of varible loci in a population. If most common allele does not exceed 0.95 or 0.99 frequency in a given population, this locus is called as polymorphic. This can be calculated by using the formula;

$$P = \frac{n_{pj}}{n_{total}}$$

where n_{pj} equals the number of polymorphic loci, n_{total} equals to total number of loci.

Observed number of alleles

It is also called allelic richness, includes the number of alleles detected at a locus. It has higher sensitivity compare to heterozygosity because of small population size (allendorf and Luikart, 2007). To calculate, the formula is as follows;

$$Na = \frac{\sum_i na_i}{r}$$

where r equals to the number of loci and na_i equals to the number of alleles detected per locus.

Effective number of alleles (Ne)

It shows the number of equally frequent alleles which is available in a population. It calculates the number of alleles which would be expected in a locus for each population (Kimura and Crow, 1964). The formula is as follows; p_i^2

$$mean(Ne) = \frac{1}{\sum p_i^2}$$

where n is equals to effective number of allele, p_i is equals to frequency of the i^{th} allele in a locus.

Heterozygosity

Heterozygosity, the fraction of individuals in a population that is heterozygous for a particular locus, is the most common tool to measure genetic diversity of a population. Alleles at higher frequencies may affect the estimated value (Nei, 1987). The formula is as follows;

$$H = \frac{2N(1-\sum x_i^2)}{(2N-1)}$$

where N is equals to number of individuals and x_i equals to the allele frequency in a SSR loci.

3.7. F statistics

F statistics show the statistically expected level of heterozygosity in a population. It measures the degree of a reduction in heterozygosity relative to Hardy-Weinberg expectations (Allendorf and Luikart, 2007). There are three indices used in F statistics;

F_{IS}, the measure of reduction of heterozygosity of an individual due to nonrandom mating within subpopulations. It ranges from -1 to 1. Negative value of F_{IS} indicates excess of heterozygosity, while positive value of F_{IS} indicates excess of homozygosity in each population. The formula is;

$$F_{IS} = 1 - \left(\frac{H_I}{H_S}\right)$$

F_{IT}, the measure of reduction of heterozygosity of an individual in relation to the total population. It ranges from -1 to 1. Negative value of F_{IT} indicates excess of heterozygosity, while positive value of F_{IT} indicates excess of homozygosity in a group of population. The formula is;

$$F_{IT} = 1 - \left(\frac{H_I}{H_T}\right)$$

F_{ST}, the degree of reduction in heterozygosity of a subpopulation due to genetic drift. It ranges from 0 to 1. As it gets close to 1, genetic differentiation among populations increases. The formula is;

$$F_{ST} = 1 - \left(\frac{H_S}{H_T}\right)$$

The genetic structure of populations can be estimated by the formula;

$$(1 - F_{IT}) = (1 - F_{IS})(1 - F_{ST})$$

where H_I represents the average observed heterozygosity in subpopulations, H_S represents the average expected heterozygosity estimated from each subpopulation and H_T represents the total gene diversity or expected heterozygosity in the total population as estimated from the pooled allele frequencies (Allendorf and Luikart, 2007).

***Number of migrants* (*Nm*)**

It determines estimated gene flow from F_{ST} for nuclear SSR markers. The formula is;

$$Nm = \frac{\frac{1}{F_{ST}} - 1}{4}$$

Where F_{ST} represents the degree of population genetic differentiation.

3.8. Population genetic structure

3.8.1. Structure analysis

Genetic structure (STRUCTURE 2.3.4; Pritchard *et al*., 2000) analysis by including data from 10 nuclear SSRs, was carried out to determine the number of genetically homogeneous groups. The structure analysis is a universal Bayesian model to determine subgroups which have distinctive allele frequencies (Evanno *et al.*, 2005). The STRUCTURE algorithm constructs genetic clusters from a collection of individual multilocus genotypes and calculates for each individual coefficients of membership in subpopulations which belong to each cluster. Due to not providing a correct estimation of the number of clusters (K), an *ad hoc* statistics (*Δ*K), the rate change in log probability of data between successive K values, is used to obtain accurate number of clusters (Evanno *et al*., 2005).

Statistics used to select K

To evaluate the K**,** *ad hoc* quantity (ΔK) was calculated. When ΔK shows the top peak it means the true value of K is obtained (Evanno *et al.*, 2005).

Ln P(D) shows the log likelihood for each K in STRUCTURE output obtained by first computing the log likelihood of the data at each step of the Markov Chain Monte Carlo(MCMC). The LnP(K) gives the mean likelihood over 10 runs for each K, the average of 10 values of Ln P(D). Steps for evaluating ΔK were as below;

1. The mean difference between successive likelihood values of K was plotted;

   $Ln'(K)=LnP(K)-LnP(K-1)$

2. First the difference between values L′(K) were calculated and then, absolute value was taken;

   $|Ln''(K)=Ln'(K+1)-Ln'(K)|$

3. Estimated ΔK as the mean of the absolute values of Ln″(K) was averaged over 10 runs and divided by the standard deviation of LnP(K);

$$\Delta K = \frac{m|Ln''(K)|}{s|LnP(K)|}$$

The numbers of clusters (K) were set from 1 to 10. The Burn-in period program starts from 5000 repeats to decrease installation effect and continue until 50000 MCMC repeats to obtain correct prediction for each clusters. It was replicated 10 times for each run at each of K=1 to 10.

To calculate the average estimated cluster membership coefficient matrices, the CLUMPP (Jakobsson and Rosenberg, 2007) was used. After CLUMPP output datum (Jakobsson and Rosenberg, 2007) obtained, it was used by DISTRUCT, to display the graphics of population structure (Rosenberg, 2004).

3.8.2. Genetic distance

Genetic distance constructed by applying coancestry identity neighbour joining method in GDA (Genetic data analysis software; Lewis and Zaykin, 2002). Distance matrix constructed distances /identity measures based on 10 loci for 10 goat willow populations. Coancestry distance values range from 0 to 1. If distance equals to 0, there is no difference between populations. Coancestry identity equals to pairwise F_{ST} (Weir and Cockerham, 1988). To construct dendogram, neighbor joining method was used.

3.8.3. Principal coordinate analysis

Principal coordinate analysis was carried out by applying covariance and standardized option of GenAlEx (Peakal and Smause, 2012).

CHAPTER 4

RESULTS

4.1. Nuclear DNA amplification

Leaves of 180 goat willow (*Salix caprea*) genotypes were sampled from North Eastern Turkey. DNA extraction from leaves was carried out successfully by using CTAB protocol. Then, PCR amplification reactions yielded clear bands which were observed by gel electrophoresis The peaks were observed by Peak Scanner Software v1.0. Population samples used for PCR amplification reactions.

10 SSR primers were analyzed to determine genetic diversity of goat willow. The photos of PCR products and their sizes were illustrated in Figures 4.1.

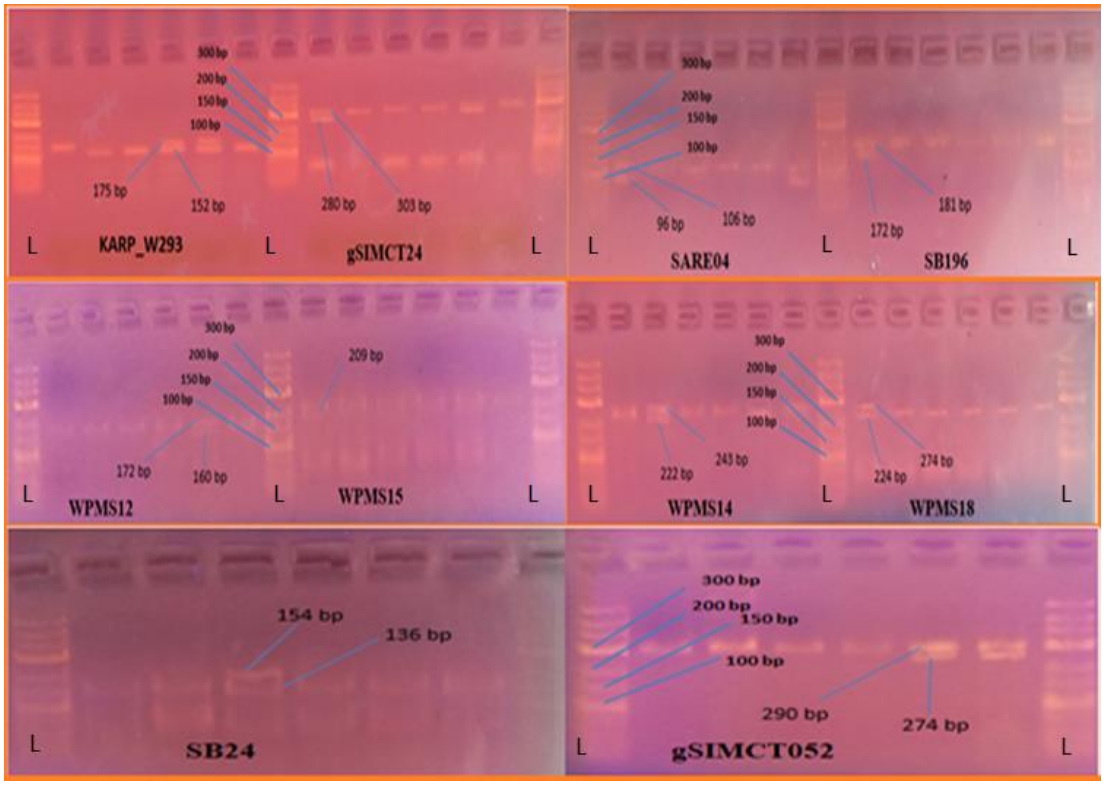

Figure 4.1 The banding patterns of primers on KARP_W293, gSIMCT24, SARE04, SB196, WPMS12, WPMS15, WPMS14, WPMS18, SB24 and gSlMCT052 agarose gel. L= DNA size ladder

4.2. Population Genetic Structure

Salix caprea was checked whether sampled genotypes were duplicated due to clonal propagation of the species by using the Genclone2 software (Arnaud-Haond *et al.*, 2007). The results from this analyses indicated that there is no clonal duplications among sampled genotypes.

When *Salix caprea* populations were checked whether their loci had null alleles by using Genepop 4.2 (Raymound and Rousset, 1995), WPMS15 and SB196 had lower than 0.05 mean values; WPMS14, SB24, WPMS12, WPMS18, GSLMCT052, GSIMCT24, KARP_W293 had between 0.05 and 0.2 mean values and SARE04 had greater than 0.2 mean values (Appendix E). Null allele value is high if null allele value is greater than 0.20 (Chapuis and Estoup, 2006).

4.2.1. Descriptive statistics of loci

According to descriptive statistics of loci, all loci were polymorphic. The observed number of alleles and their sizes were provided in Table 4.1. The observed number of alleles per locus for 10 SSR loci was moderate level with an average of 8.9. The range was between 4 in the loci WPMS12 and WPMS15 and 18 in the locus Karp_W293 (Table 4.1). Moreover, number of different alleles (Na) for loci ranged from 2.0 to 9.6 and mean number of different alleles for loci was 4.77. Besides, effective alleles for loci ranged from 1.145 to 5.982 and average mean was 2.844 (Table 4.2).

While the observed heterozygosities for single loci ranged from 0.082 to 1.000 (average: 0.503), the expected heterozygosities ranged from 0.119 to 0.827 (average: 0.538). Most of the studied loci showed lower observed heterozygosity than expected heterozygosities. The locus WPMS12 had the lowest; while, the locus WPMS15 had the highest allelic diversity values (Table 4.2).

The mean inbreeding coefficient (F_{IS}) in all loci was 0.059. Having positive F_{IS} indicates excess observed heterozygosity (Ho) compared to expected heterozygosity (He). Only WPMS15, SB24 and SB196 loci had excess observed heterozygosity (Table 4.2).

Table 4.1 The sizes of observed alleles and number of alleles for each SSR loci in *Salix caprea* populations

SSR Loci	Size of Alleles (bp)	Number of Alleles
WPMS12	158,160,164,172	4
WPMS18	218,224,274,280,286,298	6
WPMS14	212,216,219,222,225,228,231,234,237,243	10
WPMS 15	202,209,216,220	4
SARE04	86,88,90,92,94,96,98,100,102,106	10
SB24	124,126,130,133,136,139,142,145,148,154,157	11
gSlMCT052	272,274,278,280,282,284,286,288,290,292	10
SB196	163,166,172,175,178,181	6
gSIMCT24	274,280,291,297,299,301,303,305,313,315	10
KARP_W293	111,116,118,122,126,128,130,132,135,137,139,141,143,145,147, 149,152,175	18

Table 4.2 Descriptive statistics of loci parameters for goat willow populations in North Eastern Turkey for 10 loci.

Loci	N	Na	Ne	Ho	He	F_{IS}
WPMS14	18.000 ±1.832	5.200 ±0.359	3.234 ±0.207	0.600 ±0.057	0.679 ±0.020	0.117 ±0.084
WPMS15	18.000 ±1.832	2.200 ±0.200	2.018 ±0.018	1.000 ±0.000	0.504 ±0.004	-0.985 ±0.015
SB24	18.000 ±1.832	5.700 ±0.423	2.809 ±0.237	0.686 ±0.043	0.622 ±0.030	-0.108 ±0.053
SB196	17.800 ±1.960	3.900 ±0.277	1.778 ±0.130	0.469 ±0.068	0.405 ±0.051	-0.138 ±0.053
SARE04	18.000 ±1.832	6.700 ±0.423	4.190 ±0.303	0.548 ±0.044	0.750 ±0.017	0.268 ±0.062
WPMS12	18.000 ±1.832	2.000 ±0.258	1.145 ±0.035	0.082 ±0.026	0.119 ±0.028	0.253 ±0.141
WPMS18	17.800 ±1.890	2.700 ±0.260	1.570 ±0.159	0.190 ±0.031	0.323 ±0.046	0.397 ±0.120
GSLMCT052	18.000 ±1.832	4.200 ±0.646	2.138 ±0.363	0.259 ±0.051	0.437 ±0.067	0.355 ±0.100
GSIMCT24	18.000 ±1.832	5.500 ±0.342	3.576 ±0.237	0.559 ±0.032	0.707 ±0.022	0.202 ±0.053
KARP_W293	17.900 ±1.832	9.600 ±0.653	5.982 ±0.361	0.641 ±0.038	0.827 ±0.011	0.224 ±0.047
Total Mean	17.950 ±0.557	4.770 ±0253	2.844 ±0.156	0.503 ±0.029	0.538 ±0.023	0.053 ±0.046

N = Sample size, Na = Number of different alleles, Ne = number of Effective Alleles, He = Expected heterozygosity, Ho = Observed heterozygosity, F_{IS} = Fixation index

4.2.2. Descriptive statistics of populations

All populations had high polymorphism rate. In Eynesil, Kafkasor and Surmene populations, 90% loci were polymorphic. In the other populations, all loci were polymorphic. The mean proportion of polymorphic loci was 97% (Table 4.3).

The mean number of alleles (Na) was 4.77. It ranged from 3.50 (Kafkasor) to 5.6 (Sacinka). The mean number of effective alleles (Ne) was 2.844. The range was from 2.502 (Kafkasor) to 3.402 (Sacinka) (Table 4.3).

The mean observed heterozygosity (Ho) of populations ranged from 0.446 (Koprubasi) to 0.567 (Kafkasor). The mean expected heterozygosity (He) of populations ranged from 0.448 (Surmene) to 0.610 (Sacinka). Only Kafkasor and Surmene populations had higher observed heterozygosity.

Because average observed heterozygosity (0.503) was lower than average expected heterozygosity (0.538), average fixation index was positive (average: 0.053).

Table 4.3 Descriptive population genetic diversity parameters for goat willow populations in North Eastern Turkey for 10 loci.

Pop	N	%P	Na	Ne	Ho	He	F_{IS}
Eynesil	17	90.00%	4.500 ±0.687	2.804 ±0.339	0.482 ±0.091	0.572 ±0.073	0.125 ±0.154
Kafkasor	8	90.00%	3.500 ±0.601	2.502 ±0.420	0.567 ±0.117	0.497 ±0.077	-0.096 ±0.168
Surmene	18	90.00%	4.400 ±0.897	2.569 ±0.525	0.461 ±0.105	0.448 ±0.097	-0.062 ±0.124
Besikduzu	27	100.00%	5.500 ±0.820	2.870 ±0.496	0.511 ±0.084	0.555 ±0.069	0.060 ±0.134
Koprubasi	13	100.00%	3.900 ±0.605	2.683 ±0.502	0.446 ±0.096	0.496 ±0.085	0.055 ±0.151
Borcka	21	100.00%	4.900 ±0.994	2.953 ±0.600	0.495 ±0.105	0.539 ±0.081	0.125 ±0.182
Sacinka	15	100.00%	5.600 ±0.991	3.402 ±0.604	0.540 ±0.102	0.610 ±0.069	0.140 ±0.173
Yusufeli	14	100.00%	5.200 ±0.757	2.831 ±0.436	0.526 ±0.075	0.564 ±0.065	0.041 ±0.147
Iskenderli	23	100.00%	5.500 ±0.847	3.183 ±0.666	0.526 ±0.071	0.562 ±0.074	0.001 ±0.114
Hattila	24	100.00%	4.700 ±0.790	2.644 ±0.424	0.478 ±0.087	0.532 ±0.068	0.117 ±0.141
Total Mean	17.950 ±0.557	97.00% ±1.53%	4.770 ±0.253	2.844 ±0.156	0.503 ±0.029	0.538 ±0.023	0.053 ±0.046

N = Sample size, %P = Proportion of polymorphic loci, Na = Number of different alleles, Ne = number of Effective Alleles, Ho = Observed heterozygosity, He = Expected heterozygosity, F_{IS} = Fixation index

Allelic richness

Private allele is a good indicator to detect the genetic diversity of populations. For all population of goat willow in North Eastern Turkey, there were 14 private alleles. On the other hand, Eynesil and Yusufeli populations had three of these private alleles, Surmene, Besikduzu, Sacinka and Iskenderli had two of these private alleles. Kafkasor, Koprubasi, Borcka and Hattila populations had no private allele (Table 4.4).

Table 4.4 Private alleles of *Salix caprea* populations in North Eastern Turkey

Pop	Locus	Allele	Freq
Eynesil	WPMS14	212	0.029
Eynesil	WPMS18	280	0.235
Eynesil	GSIMCT24	313	0.059
Surmene	WPMS14	216	0.028
Surmene	GSIMCT24	274	0.056
Besikduzu	SB24	157	0.019
Besikduzu	SARE04	100	0.019
Sacinka	WPMS14	237	0.067
Sacinka	SB24	154	0.033
Yusufeli	SB24	148	0.036
Yusufeli	WPMS18	286	0.125
Yusufeli	KARP_W293	122	0.071
Iskenderli	WPMS15	187	0.022
Iskenderli	WPMS15	190	0.022

4.2.3. F statistics

The F statistics are used to analyze the structures of subdivided populations. Moreover, it can be used to check the expected degree of a reduction in heterozygosity when compared to Hardy–Weinberg expectation.

Positive value of inbreeding coefficient F_{IS} shows heterozygote deficiency, which means observed heterozygosity values for these populations or these loci are lower than expected heterozygosity and so homozygosity level is higher. Average mean value of F_{IS} in populations was 0.053. This means, within the populations, heterozygotes were 5.3% lower than expected (Table 4.5). Only Kafkasor and Surmene populations had negative F_{IS} values. Only WPMS15, SB24 and SB196 loci had negative values. Therefore, their heterozygosity levels were higher. Average mean value of the inbreeding coefficient (F_{IS}) in loci was 0.071. These values

indicated that within the loci, heterozygotes were 7.1% lower than expected (Table 4.5).

F_{IT} describes total inbreeding coefficient of individuals within subpopulations. Its mean value was 0.128, which means 12.8% excess of homozygotes was observed within populations. The loci WPMS15, SB24 and SB196 had negative F_{IT} value showing higher heterozygosity than expected (Table 4.5).

The mean F_{ST} values for all populations were found to be 0.075 (Table 4.5). There was 7.5 % genetic differentiation among populations. This result shows that a moderate level of differentiation occurs among these populations. The WPMS18 and GSlMCT052 loci contributed differentiation of *Salix caprea* populations mostly. On the other hand, WPMS15 did not contribute differentiation at all.

The mean *Nm* values for all populations found to be 72.209 (Table 4.5). There was a little differentiation among populations. WPMS15 had the highest *Nm* values, found equally in all populations.

Table 4.5 F statistics for each locus.

	F_{IS}	F_{IT}	F_{ST}	*Nm*
WPMS14	0.116	0.175	0.066	3.528
WPMS15	-0.984	-0.983	0	740.833
SB24	-0.102	-0.065	0.034	7.105
SB196	-0.157	-0.08	0.067	3.486
SARE04	0.271	0.328	0.078	2.954
WPMS12	0.315	0.346	0.045	5.282
WPMS18	0.411	0.514	0.176	1.173
GSLMCT052	0.407	0.489	0.138	1.568
GSIMCT24	0.21	0.272	0.079	2.932
KARP_W293	0.225	0.281	0.072	3.230
Mean	0.071 ± 0.132	0.128 ±0.139	0.075 ±0.016	77.209±73.738

F_{IS} = The fixation index within subpopulations, F_{IT}= The fixation index over total population, F_{ST} = The reduction in fixation index due to differences among subpopulations, Nm= the number of migrants entering a population per generation (Nei, 1987).

Only populations from east of Trabzon province (Surmene and Koprubasi) had negative F_{IS} (-0.003). F_{ST} value among populations from Artvin province was highest (0.055) compare to among populations from west of Trabzon province and east of Trabzon province. For all three clusters F_{ST} value was lower than 0.05 which indicates low differentiation. *Nm* value was highest in the populations from east of Trabzon province (47.252). For all three clusters *Nm* value was higher than 1 which indicates high migration rate among populations.

Table 4.6 F statistics for Clusters

Region	Populations	F_{IS}	F_{IT}	F_{ST}	Nm
East of Trabzon province	Eynesil Iskenderli Besikduzu	0.067 ±0.125	0.098 ±0.131	0.045 ±0.024	47.252 ±29.631
West of Trabzon province	Surmene Koprubasi	-0.003 ±0.131	0.022 ±0.134	0.030 ±0.011	13.112 ±3.003
Artvin province	Kafkasor Borcka Yusufeli Sacinka Hattila	0.075 ±0.146	0.116 ±0.148	0.055 ±0.011	4.019 ±0.619

F_{IS} = The fixation index within subpopulations, F_{IT}= The fixation index over total population, F_{ST} = The reduction in fixation index due to differences among subpopulations, Nm= the number of migrants entering a population per generation (Nei, 1987).

4.4. Population Structure of *Salix caprea*

The analysis of log likelihood values across STRUCTURE (Pritchard *et al*., 2000; Falush *et al*., 2003, Falush *et al*., 2007) runs using ΔK suggested the presence of two main groups (Table 4.6). Pritchard *et al*. (2000) pointed out that "*We may not always be able to know the TRUE value of K, but we should aim for the smallest value of K that captures the major structure in the data*" . The description viable for the result.

Table 4.7 STRUCTURE analyses of 10 nuclear SSR markers for 180 *Salix caprea* genotypes.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	ΔK
1	10	-4789.26	0.29	—	—	—
2	10	-4643.81	3.17	145.45	60.17	18.94
3	10	-4558.53	7.95	85.28	36.96	4.65
4	10	-4510.21	5.82	48.32	63.22	10.85
5	10	-4525.11	124.57	-14.90	51.81	0.42
6	10	-4488.20	32.12	36.91	9.13	0.28
7	10	-4460.42	25.78	27.78	93.44	3.62
8	10	-4526.08	130.28	-65.66	17.59	0.14
9	10	-4574.15	243.99	-48.07	157.21	0.64
10	10	-4779.43	593.68	-205.28	—	—

Magnitude of *Δ*K as a function of *K* calculated using *S. caprea* genotypic data. Runs were performed for K=1 to 10. (K : The number of studied populations, LnP(K): The posterior probability of the data for a given K, Stdev LnP(K): Standard deviation, Ln'(K): The ratio of change of the likelihood function according to K, |Ln(K)''|: Second order rate of change of L'K, *Δ*K: The number of genetically homogeneous groups)

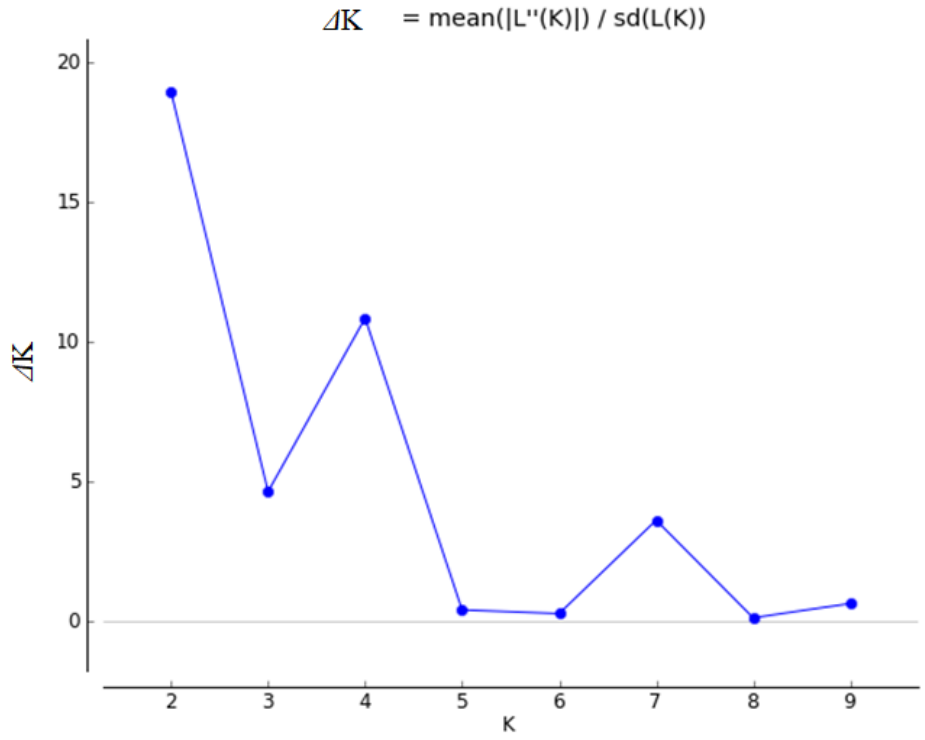

Figure 4.2 *Δ*K versus K graph (*Δ*K: The number of genetically homogeneous groups, K : The number of studied populations)

For the two determined clusters, the probabilities of membership in each cluster were aligned using CLUMPP 1.1.2 (Jakobsson and Rosenberg 2009) with full search option. The first cluster included all Artvin populations except Kafkasor. The second cluster included populations from Trabzon and Kafkasor. Yusufeli had the highest membership value with 0.89 and Hattila had the lowest value with 0.57 for the first cluster while Surmene and Koprubasi had the highest value with 0.89 and Kafkasor had the lowest value with 0.55 for the second cluster (Table 4.7).

There was a distinct differentiation between two groups except Kafkasor and Hattila populations. Genetic and geographic distances were associated for 10 populations. The populations from Trabzon region were in the same cluster while the populations from Artvin region were in the cluster.

Table 4.7. The ratio of estimated membership values of the two inferred genetic groups.

***Salix Caprea* Populations**	**The estimated membership values**		**Number of Individuals**
	Cluster 1	**Cluster 2**	
Eynesil	0.12	0.88	17
Surmene	0.11	0.89	18
Koprubasi	0.11	0.89	13
Besikduzu	0.18	0.82	27
Iskenderli	0.27	0.73	23
Kafkasor	0.45	0.55	8
Hattila	0.57	0.43	24
Borcka	0.81	0.19	21
Yusufeli	0.89	0.11	14
Sacinka	0.83	0.17	15

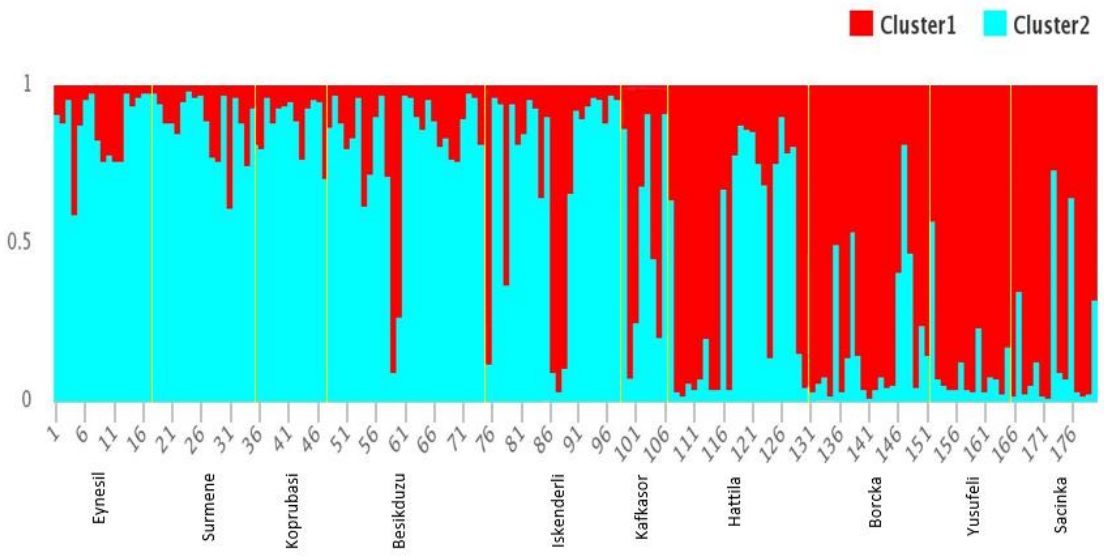

Figure 4.3 STRUCTURE analyses for 180 *Salix caprea* genotypes from 10 populations according to nuclear SSR markers in Eastern Black Sea Region. Distribution of STRUCTURE defined groups among 10 populations for *K* = 2, each population's membership coefficients were averaged to generate *Q*-matrix. Red and blue colors present group 1, group 2 accordingly.

In figure 4.3, two clusters and their association with populations are indicated. The clusters were estimated by the STRUCTURE run of the highest estimated probability. Each cluster has a specific color. Yellow segments classify the populations. The populations were divided into 2 colored sections which correspond to the membership coefficients in the subgroups.

4.5. Genetic distance of *Salix caprea* populations

Two major clusters were formed on the dendogram according to GDA results. One cluster was consisted of populations from Trabzon province, except for the Kafkasor population and other cluster was consisted of populations from Artvin province. There was a little differentiation between these two clusters (Figure 4.4).

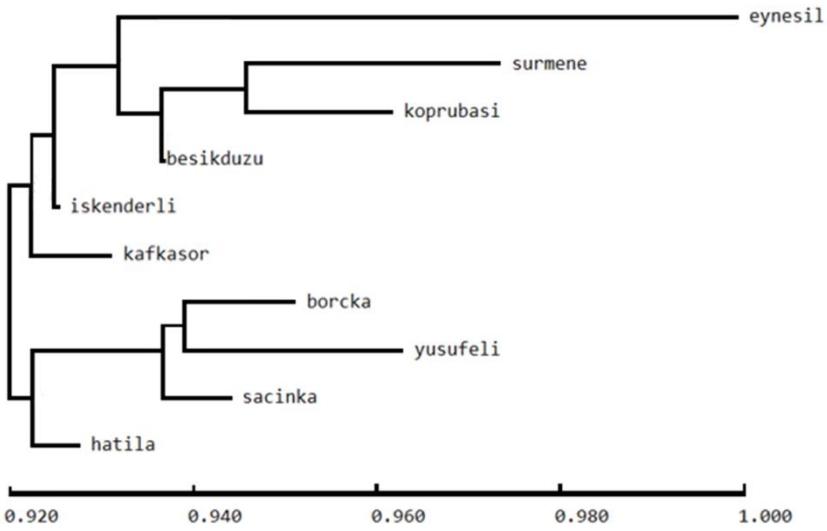

Figure 4.4 Dendogram was formed by applying coancestry identity for 10 *Salix caprea* populations with neighboor joining method.

4.6. Principal coordinate analysis

PcoA matrix constructed using genetic covariance matrices based on 10 loci for 10 goat willow populations.

The results were similar to STRUCTURE results (Figure 4.5). Populations mainly divided into two groups. Besikduzu and Iskenderli populations were very close to each other. In addition, Sacinka population was placed distantly from Borcka, Yusufeli and Hattila populations as well as Eynesil and Kafkasor populations were also located distantly from Koprubasi, Iskenderli, Sürmene, Besikduzu populations (Figure 4.5).

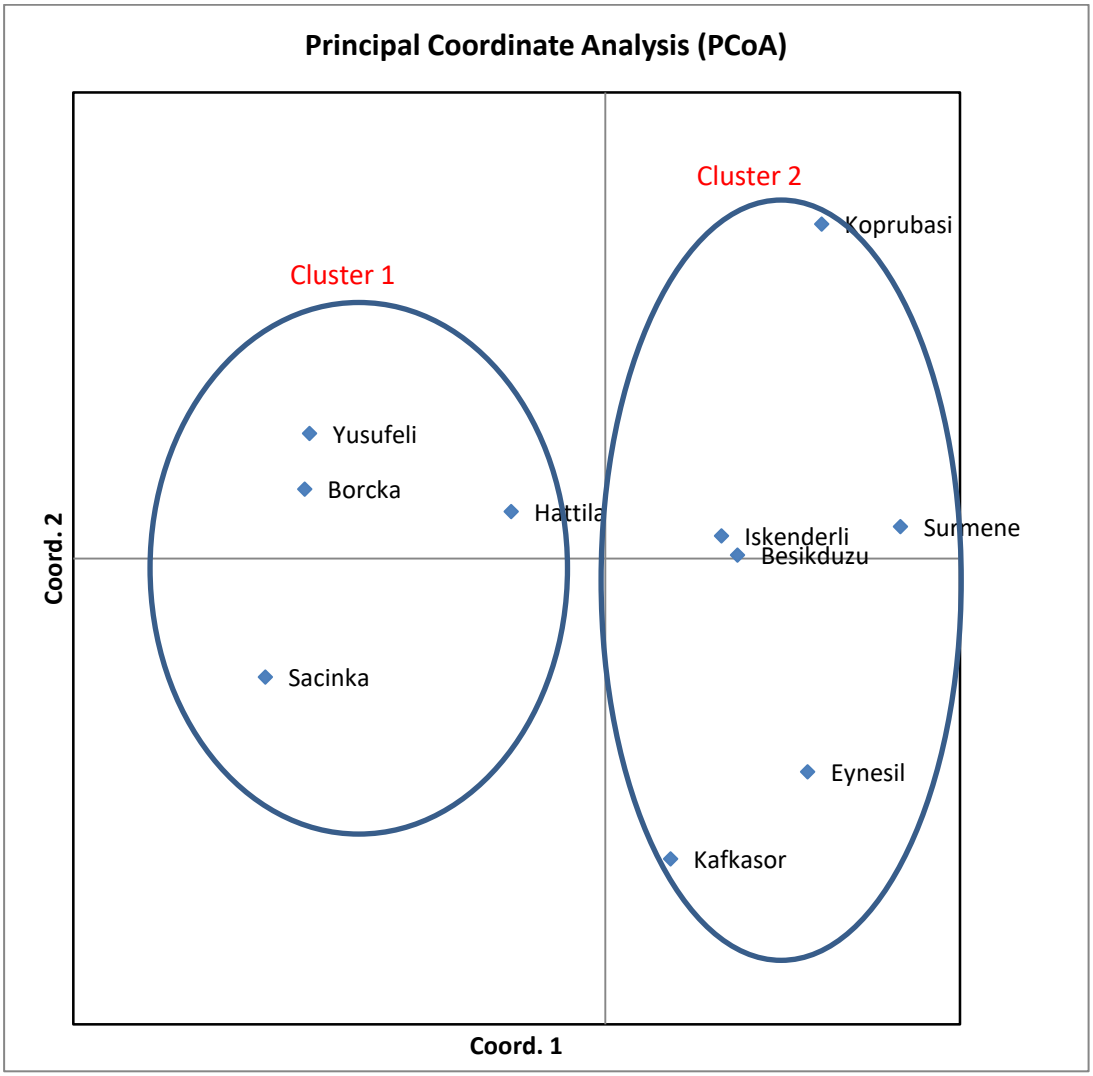

Figure 4.5 Principal coordinate Analysis (PcoA) results for 10 populations of *Salix caprea*

CHAPTER 5

DISCUSSION

5.1. Genetic diversity of *Salix caprea* populations in Eastern Black Sea Region

There were no genotypes with same alleles which mean clonal duplication did not occur among the sampled 180 *Salix caprea* genotypes. *S. caprea* (goat willow) is a species that is recalcitrant with natural vegetative regeneration according to Perdereau (2014). It grows alone and does not form stand. Moreover, human influence on goat willows' distribution is negligible. Therefore, it was expected that clonal propagation would be low ratio.

Ten loci were checked whether they were null alleles by using genepop 4.2 (Raymound and Rousset, 1995). According to Chapuis and Estoup (2006), loci would be categorized into 3 classes pursuant to null allele frequency: negligible if null allele mean vaue is lower than 0.05, moderate if null allele mean value is between 0.05 and 0.20 and large if null allele value is bigger than 0.20. Therefore, nine loci did not have null allele and did not affect homozygosity. The SARE04 loci might be affected by null alleles. Since observed heterozygosity (Ho) – expected heterozygosity (He) differences were also high. Therefore, to check whether null allele of SARE04 loci affected the results, analyses were done after removing SARE04 from the dataset. The results did not change much. Moreover, when null allele checked for population level, Kafkasor population had negligible (0.00) while Borcka (0.16), Sacinka (0.13) and Hattila (0.16) had low level of null allele values for SARE04 loci which means, populations from Artvin province had acceptable null allele results. Furthermore, although KARP_W293 and GSLMCT052 loci had higher Ho – He differences, their null allele values were low. These two indications support the idea that the locus SARE04 did not affected by null alleles. Thus, having

high Ho – He differences was not the result of null allele but it was cause of null allele. Therefore, the locus was added to the analyses and results as well.

Allelic richness in studied populations

Allele diversity is an important parameter to asses genetic diversity (Kalinowski, 2004). As allelic diversity increases, the genetic diversity also increases in populations. According to El Mousadik and Petit (1996) allelic richness is more important than heterozygosity for measuring diversity, particularly for genetic conservation.

WPMS14, WPMS18, GSIMCT24, SB24, SARE04, KARP_W293, WPMS15 loci had the private alleles. Eynesil and Yusufeli populations had the highest number of private alleles. If the species conservation of genetic resources of the species is concerned, populations having high private alleles should be protected. Moreover, populations having higher private alleles could also be protected if it is needed to conduct new cultivation programmes.

In this study, mean observed and effective number of alleles per locus in studied populations were highly different. The highest observed (5.6) and effective (3.4) number of alleles were observed in the Sacinka population. On the other hand, Kafkasor population had the lowest observed (3.5) and effective (2.5) allele number. The reason for having low effective number of alleles may be due to low population sizes. *S. caprea* had low density in sampled locations. It was hard to find *S. caprea* in same locations during sampling. Low density distribution is a natural characteristic of goat willow. In similar studies, only 170 genotypes were sampled from Austuria, Czech Republic, Slovenia (Puschenreiter *et al.*, 2010) and 183 genotypes were sampled from whole Ireland (Perdereau *et al.*, 2014).

Proportion of polymorphic loci

Proportion of polymorphic loci is used to show locus that has variation. WPMS12, WPMS15, WPMS18 and SB196 loci showed low level of polymorphism compared to other primers; four – four – six – six allelic region respectively. WPMS14, SARE04, SB24, gSIMCT052 and gSIMCT24 loci showed moderate level of polymorphism; ten – ten – eleven – ten – ten allelic region, respectively. KARP_W293 locus had the highest level of polymorphism; eighteen polymorphic region. WPMS12, WPMS18, WPMS14,WPMS15 and KARP_W293 SSR markers were designed for *Populus* species and SARE04, SB24, gSIMCT052, SB196 and gSIMCT24 SSR markers were designed for *Salix alba/excelsa* species. Both *Populus* and *Salix alba/excelsa* SSR markers contributed polymorphism equally for *Salix caprea* populations studied in this research. To conclude, WPMS14, SARE04, SB24, gSIMCT052, gSIMCT24 and especially KARP_W293 primers would be good SSR markers to study genetic diversity of *Salix caprea.*

All studied populations were highly polymorphic. Proportion of polymorphic loci was calculated as 100% for all populations except Eynesil, Kafkasor and Surmene populations which had 90%.

Heterozygosity

The mean observed heterozygosity values lower than the expected heterozygosity values for most of the loci; WPMS15, SB24 and SB196 loci had higher observed heterozygosity (Ho) values but other seven loci had lower Ho values compared to expected heterozygosities (He). Observed heterozygosities of the loci ranged from 0.082 (WPMS12) to 1.000 (WPMS15) and He ranged from 0.119 (WPMS12) to 0.827 (KARP_W293).

The mean observed heterozygosity values lower than the expected heterozygosity values for most of the populations, only Kafkasor and Surmene populations had higher observed heterozygosity values. Moreover, while observed heterozygosity (Ho) values were ranged from 0.446 to 0.567, expected heterozygosity (He) values was ranged from 0.448 to 0.610. In the study of Perdereau (2014), genetic diversity

of *S. caprea* in Irish populations, the SSR results were similar. In their populations, observed heterozygosity was ranged from 0.32 to 0.51. Beside this, their populations, also had lower Ho value compared to He value (mean Ho= 0.41 and mean He= 0.49). The high rate of homozygosity found in these populations could be explained with a small effective population size. Genetic drift might also cause increment of homozygosity. Moreover, range of mountains found in Black sea region have an isolation effect on populations and that could increase homozygosity.

When the Hardy-Weinberg law was considered; if Ho is higher than He, inbreeding is not expected and genetic diversity in the population is in required level. On the other hand, when mean observed and expected heterozygosity values of population were considered (mean Ho= 0.503, mean He= 0.538), goat willow populations in the North Eastern Turkey had about 3,5 % inbreeding.

5.2. Genetic differentiation of *Salix caprea* populations in Eastern Black Sea Region

F statistics

The mean F_{IS}, fixation coefficients, of populations was 0.053. This indicates that observed heterozygosity was lower than expected heterozygosity for goat willow populations, which means, there was a slight excess of homozygotes for all populations. Only Kafkasor (-0.096) and Surmene (-0.062) populations had negative F_{IS} values. The positive F_{IS} of the goat willow populations indicated a loss of genetic diversity, which reflects deviations from Hardy–Weinberg equilibrium. These excess of homozygotes relative to Hardy-Weinberg proportions could result from inbreeding within local populations. (Allendorf and Luikart, 2007; Perdereau 2014).

The mean F_{IS} of 10 loci was 0.071 which is the indicator of the slight excess of homozygosity. Only three of the loci had negative F_{IS}; WPMS15, SB24 and SB196. Positive loci could be under selection pressure by natural selection or breeding selection. The slight positive F_{IS} result for loci also indicates a deviation from HWE.

It has been accepted that the value ranges between 0 and 0.05 suggests low genetic differentiation; between 0.05 and 0.15 suggests moderate differentiation; between

0.15 and 0.25 suggests great differentiation; and above 0.25 suggests very great genetic differentiation (Hartl & Clark, 1997). F_{ST} value for 10 populations was found to be 0.075 which is moderate. This result showed that a moderate level of differentiation occurs among populations; 7.5 % of total genetic variation of populations was between population and 92.5% of total genetic variation was within populations. The WPMS18 locus was represented for great differentiation among populations (F_{ST} =0.176). On the other hand, WPMS15 did not cause differentiation among populations (F_{ST}=0.000). Perdereau (2014) reported F_{ST} as 0.16 which is nearly twice as high. Although population sizes (183 samples) are similar with this study, the difference might cause by virtue of sampling location, sampled in Ireland, and using different microsatellites markers. For example, 6 microsatellites markers were used in his research and five of them are different than these used in this study. Beside this, in another study which is related to differentiation of metallicolous and non-metallicolous *Salix caprea* populations based on phenotypic characteristics and nuclear microsatellite (SSR) markers (Puschenreiter *et al.*, 2010), F_{ST} was found as 0.0143. 11 loci was used and four of them were the same with these in this study. This F_{ST} was very low compare to this study as well as Perdereau (2014). For *S. caprea,* low variation among populations could be caused by efficient gene flow. *S. caprea* is an outcrossing, dioecious species, causing lower genetic differentiation. Moreover, due to effective cross pollination especially by wind, the level of genetic differentiation could be low.

Nm, number of migrants, is used to measure gene flow between populations. *Nm* depends on F_{ST}; as F_{ST} decreases *Nm* increases because low differentiation between populations means higher levels of gene flow. If *Nm* is bigger than 1.0, there is a constant gene flow among populations (Perdereau *et al.*, 2014). For all loci, *Nm* was higher than 1 which showed high gene flow among populations for these loci. Mean *Nm* was 77.209 which reflects a permanent gene flow among populations.

When three clusters (populations from east and west of Trabzon province and populations from Artvin province) were compared according to their F_{IS}, F_{ST} and *Nm* values, the results were close to each other. Populations from west of Trabzon province had negative F_{IS} values, indicating higher observed heterozygosity bu the

other two clusters had slight positive F_{IS}, indicating higher expected heterozygosity. All three clusters had low F_{ST} and high *Nm* values indicating low differentiation and high gene flow. Populations from east of Trabzon province had higher *Nm.* The reason for that could be they were close to each other compare to other two clusters. Therefore geographic closeness increase gene flow between these three populations.

Salix caprea had moderate level of heterozygosity value with 0.503 for the studied populations in North Eastern Turkey. Moreover, F_{ST} value was also moderate with 0.075. Goat willow distribution is generally along the riverside. Many of hydroelectric plants were built on both Coruh and Manahoz rivers from which populations studied. Some hydroelectric plant constructions are still in progress. This means that some of the *Salix caprea* populations would be under water in near future. Moreover, hydroelectric plants may change local climate which would affect *Salix caprea* negatively by increasing average annual temperature (Gyau-Boakye, 2001). Moreover, some *Salix caprea* plants natural habitats were disturbed frequently by alpine tourism activities. These factors affect *Salix caprea* species adversely. Although it is not found IUCN Red List of Threatened Species and defined as least concern in the "The Vascular Plant Red Data List for Great Britain" study (Cheffings & Farrell, 2005), *Salix caprea* might be endangered in the near future in Turkey. Therefore, conservation program should be developed to protect its distribution in North Eastern Turkey.

5.3. Population structure and landscape genetics

Structure Analysis

Population structure analysis result showed the presence of two genetically separated groups. First cluster included Borcka, Yusufeli, Hattila, and Sacinka which were from Artvin region. Moreover, second cluster included Eynesil, Surmene, Koprubasi, Besikduzu, Iskenderli and Kafkasor populations. These populations were from Trabzon region except Kafkasor. Kafkasor had 0.55 and Hattila had 0.57 membership coefficient value. Others had >73 membership coefficient value. The clustering also showed a low level of admixture within these two groups (Figure 4.3). A clear spatial separation was observed between these two clusters. Bayesian

analysis showed that the studied populations were separated according to their location. This can be explained with that gene flow ,by pollen and seed dispersal, was effective along the river bank but not so effective to cross the mountains by wind and water. There were a clear geographic isolation by force of mountain ranges between Trabzon region and Artvin region except Kafkasor and Hattila populations. Kafkasor population was close to Artvin city. Moreover, it consisted of only eight samples which could be also the reason of being not properly located among clusters. Therefore, human integrated migration of willow trees for Kafkasor population could cause to closeness of this population to both formed clusters.

According to principal coordinate analysis and dendogram results, there was also two main groups; one group consisted of Yusufeli, Borcka, Sacinka, Hattila, sampled from Artvin region and other group consisted of Koprubasi, Iskenderli, Surmene, Kafkasor, Besikduzu and Eynesil, sampled from Trabzon region except Kafkasor population. Koprubasi and Surmene populations were close to Manahoz river; Borcka, Yusufeli and Sacinka populations were close to Coruh river. Therefore, it can be concluded that rivers affect populations' distribution. Clusters and subclusters occurred by geographic barriers and being near same riversides. According to dendogram, genetically close populations were also geographically close.

Overall, although distance between populations changed, main clusters were always same for all of three methods. Trabzon populations were formed one cluster and Artvin populations were formed the other cluster. The only exception was the Kafkasor population.

In conclusions, geographic isolation and mountain ranges seem to affect genetic diversity pattern among populations as well as natural distribution of *Salix caprea* species. Moreover, The Coruh and Manahoz rivers also had an impact on the distribution of the species, species distribute on the same river were genetically close.

CHAPTER 6

CONCLUSION

The results of this study showed that 10 nuclear SSR markers provided sufficient resolution to analyze population genetic structure of *S. caprea* populations in North Eastern Turkey.

In this study, the level of genetic diversity was shown for 10 goat willow populations in North Eastern Turkey. When compared all the populations according to their expected and observed heterezoygosity, number of alleles, polymorphic loci ratio among populations, significant differences were not observed. Genetic diversity of all populations is generally in the moderate level. By this study, breeding studies for later generation of goat willow populations would be done to keep the desired level of genetic diversity and adaptability to different ecological environments.

In terms of population genetic structure study, two major geographically and genetically distinct groups were obtained. The first cluster included goat willow populations from Artvin region. Second cluster was populations from Trabzon region except Kafkasor. This result is similar with dendogram result as well as Principal coordinate analysis. Geographic isolation of the 10 studied populations caused formation of the two genetic clusters.

Due to *Salix caprea* geographic dispersal near to river, construction of dams to these regions might endanger genetic diversity of *Salix caprea* in near future. Moreover, the study might also expand to the other regions to identify whole genetic diversity of *S. caprea* over Turkey.

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APPENDIX A

THE COLLECTED LOCATIONS OF *SALIX CAPREA* POPULATIONS

Table A.1

Region	City	Code	North	East	Altitude
Eynesil	Giresun	EYN1	41.04562	39.11444	131
Eynesil	Giresun	EYN2	41.03232	39.11839	347
Eynesil	Giresun	EYN3	41.03167	39.11877	358
Eynesil	Giresun	EYN4	40.99942	39.12681	623
Eynesil	Giresun	EYN5	40.99886	39.12585	635
Eynesil	Giresun	EYN6	40.99622	39.12603	642
Eynesil	Giresun	EYN7	40.99533	39.12648	642
Eynesil	Giresun	EYN8	40.99638	39.12407	674
Eynesil	Giresun	EYN9	40.98743	39.13086	785
Eynesil	Giresun	EYN10	40.98405	39.13756	908
Eynesil	Giresun	EYN11	40.98463	39.13818	911
Eynesil	Giresun	EYN12	40.98811	39.13556	721
Eynesil	Giresun	EYN13	40.99204	39.14377	864
Eynesil	Giresun	EYN14	40.99498	39.14492	872
Eynesil	Giresun	EYN15	40.9986	39.14661	832
Eynesil	Giresun	EYN16	40.99906	39.14716	831
Eynesil	Giresun	EYN17	41.00166	39.14531	770
Kafkasor	Artvin	KAF1	41.17306	41.80806	867

Table A.1 (Continued)

Region	City	Code	North	East	altitude
Kafkasor	Artvin	KAF2	41.17139	41.80861	861
Kafkasor	Artvin	KAF3	41.17	41.81111	887
Kafkasor	Artvin	KAF4	41.16806	41.8125	869
Kafkasor	Artvin	KAF5	41.16833	41.81056	925
Kafkasor	Artvin	KAF6	41.16861	41.8075	966
Kafkasor	Artvin	KAF7	41.16861	41.80528	985
Kafkasor	Artvin	KAF8	41.16722	41.80583	977
Surmene	Trabzon	SU1	40.90783	40.22293	352
Surmene	Trabzon	SU2	40.9095	40.2249	317
Surmene	Trabzon	SU3	40.91117	40.22472	311
Surmene	Trabzon	SU4	40.91217	40.22269	292
Surmene	Trabzon	SU5	40.91303	40.2214	285
Surmene	Trabzon	SU6	40.91475	40.21843	269
Surmene	Trabzon	SU7	40.91517	40.21684	238
Surmene	Trabzon	SU8	40.91626	40.21608	242
Surmene	Trabzon	SU9	40.91735	40.21521	213
Surmene	Trabzon	SU10	40.91882	40.21614	190
Surmene	Trabzon	SU11	40.91997	40.21444	160
Surmene	Trabzon	SU12	40.92207	40.2163	134
Surmene	Trabzon	SU13	40.92314	40.21626	121
Surmene	Trabzon	SU14	40.92274	40.21689	130
Surmene	Trabzon	SU15	40.92312	40.21851	115

Table A.1 (Continued)

Region	City	Code	North	East	altitude
Surmene	Trabzon	SU16	40.92353	40.21861	107
Surmene	Trabzon	SU17	40.92371	40.22028	97
Surmene	Trabzon	SU18	40.92421	40.22116	66
Besikduzu	Trabzon	BES1	41.04498	39.2384	35
Besikduzu	Trabzon	BES2	41.03382	39.23422	91
Besikduzu	Trabzon	BES3	41.03308	39.23321	86
Besikduzu	Trabzon	BES4	41.02185	39.23155	157
Besikduzu	Trabzon	BES5	41.02108	39.23127	169
Besikduzu	Trabzon	BES6	41.01962	39.22853	192
Besikduzu	Trabzon	BES7	41.01913	39.22782	193
Besikduzu	Trabzon	BES8	41.01851	39.22724	189
Besikduzu	Trabzon	BES9	41.01752	39.22828	207
Besikduzu	Trabzon	BES10	41.01476	39.22921	226
Besikduzu	Trabzon	BES11	41.01256	39.23167	238
Besikduzu	Trabzon	BES12	41.00832	39.2345	270
Besikduzu	Trabzon	BES13	41.00693	39.23479	274
Besikduzu	Trabzon	BES14	41.00428	39.23543	296
Besikduzu	Trabzon	BES15	41.00322	39.23472	277
Besikduzu	Trabzon	BES16	41.00217	39.23426	270
Besikduzu	Trabzon	BES17	41.00408	39.23579	311
Besikduzu	Trabzon	BES18	41.00425	39.23625	328
Besikduzu	Trabzon	BES19	41.03362	39.2854	253

Table A.1 (Continued)

Region	City	Code	North	East	Altitude
Besikduzu	Trabzon	BES20	41.03188	39.28457	276
Besikduzu	Trabzon	BES21	41.03251	39.28428	279
Besikduzu	Trabzon	BES22	41.033	39.28306	250
Besikduzu	Trabzon	BES23	41.03201	39.28714	213
Besikduzu	Trabzon	BES24	41.03521	39.28772	175
Besikduzu	Trabzon	BES25	41.0369	39.28788	170
Besikduzu	Trabzon	BES26	41.03736	39.28965	91
Besikduzu	Trabzon	BES27	41.0388	39.29028	65
Koprubası	Trabzon	KPB1	40.80633	40.12343	322
Koprubası	Trabzon	KPB2	40.80337	40.12349	343
Koprubası	Trabzon	KPB3	40.80289	40.12358	342
Koprubası	Trabzon	KPB4	40.80479	40.13525	395
Koprubası	Trabzon	KPB5	40.80389	40.13732	395
Koprubası	Trabzon	KPB6	40.80162	40.14183	423
Koprubası	Trabzon	KPB7	40.8028	40.14268	457
Koprubası	Trabzon	KPB8	40.8031	40.14068	410
Koprubası	Trabzon	KPB9	40.80592	40.13198	362
Koprubası	Trabzon	KPB10	40.80585	40.13092	365
Koprubası	Trabzon	KPB11	40.80551	40.1297	370
Koprubası	Trabzon	KPB 1b	40.80607	40.12729	348
Koprubası	Trabzon	KPB 2b	40.80574	40.12859	356
Koprubası	Trabzon	KPB 3b	40.80553	40.13072	370

Table A.1 (Continued)

Region	City	Code	North	East	Altitude
Koprubası	Trabzon	KPB 4b	40.8016	40.12379	346
Borcka	Artvin	BOR1	41.34819	41.7333	357
Borcka	Artvin	BOR2	41.34492	41.74043	358
Borcka	Artvin	BOR3	41.33793	41.75898	403
Borcka	Artvin	BOR4	41.33573	41.76234	423
Borcka	Artvin	BOR5	41.33002	41.77053	453
Borcka	Artvin	BOR6	41.32753	41.77245	490
Borcka	Artvin	BOR7	41.32505	41.77825	526
Borcka	Artvin	BOR8	41.34819	41.79639	707
Borcka	Artvin	BOR9	41.36541	41.81304	1037
Borcka	Artvin	BOR10	41.36682	41.8166	1061
Borcka	Artvin	BOR11	41.36782	41.81825	1114
Borcka	Artvin	BOR12	41.3689	41.81893	1130
Borcka	Artvin	BOR13	41.36983	41.81945	1149
Borcka	Artvin	BOR14	41.36834	41.81749	1168
Borcka	Artvin	BOR15	41.36864	41.81408	1206
Borcka	Artvin	BOR16	41.37053	41.81706	1241
Borcka	Artvin	BOR17	41.3714	41.81795	1252
Borcka	Artvin	BOR18	41.37191	41.82245	1288
Borcka	Artvin	BOR19	41.373	41.82628	1321
Borcka	Artvin	BOR20	41.36728	41.82565	1340
Borcka	Artvin	BOR21	41.3664	41.82656	1343

Table A.1 (Continued)

Region	City	Code	North	East	Altitude
Borcka	Artvin	BOR22	41.36566	41.82978	1361
Borcka	Artvin	BOR23	41.36645	41.83321	1391
Sacinka	Artvin	SAC1	41.20182	41.90437	1187
Sacinka	Artvin	SAC2	41.1976	41.90518	1244
Sacinka	Artvin	SAC3	41.194	41.90147	1346
Sacinka	Artvin	SAC4	41.19351	41.90597	1334
Sacinka	Artvin	SAC5	41.19236	41.9022	1343
Sacinka	Artvin	SAC6	41.19289	41.90899	1387
Sacinka	Artvin	SAC7	41.19252	41.90716	1394
Sacinka	Artvin	SAC8	41.19215	41.90178	1416
Sacinka	Artvin	SAC9	41.19258	41.91258	1483
Sacinka	Artvin	SAC10	41.19067	41.91116	1504
Sacinka	Artvin	SAC11	41.19036	41.90688	1542
Sacinka	Artvin	SAC12	41.18721	41.90687	1622
Sacinka	Artvin	SAC13	41.19035	41.91773	1721
Sacinka	Artvin	SAC14	41.19148	41.8952	1300
Sacinka	Artvin	SAC15	41.18938	41.89313	1363
Yusufeli	Artvin	YUS1	41.00005	41.43821	1308
Yusufeli	Artvin	YUS2	41.00116	41.43423	1279
Yusufeli	Artvin	YUS3	41.00445	41.43002	1268
Yusufeli	Artvin	YUS4	41.00686	41.4268	1237
Yusufeli	Artvin	YUS5	41.00848	41.42763	1268

Table A.1 (Continued)

Region	City	Code	North	East	altitude
Yusufeli	Artvin	YUS6	41.01949	41.41935	1406
Yusufeli	Artvin	YUS7	41.02504	41.42229	1448
Yusufeli	Artvin	YUS8	40.97729	41.42287	1124
Yusufeli	Artvin	YUS9	40.97265	41.40998	1162
Yusufeli	Artvin	YUS10	40.92043	41.35266	1341
Yusufeli	Artvin	YUS11	40.90884	41.3456	1396
Yusufeli	Artvin	YUS12	40.90339	41.3451	1423
Yusufeli	Artvin	YUS13	40.898	41.34472	1446
Yusufeli	Artvin	YUS14	40.89253	41.3457	1471
Yusufeli	Artvin	YUS15	40.88417	41.34508	1485
Yusufeli	Artvin	YUS16	40.88095	41.34676	1506
Iskenderli	Trabzon	ISK1	40.92958	39.24319	726
Iskenderli	Trabzon	ISK2	40.93149	39.2444	748
Iskenderli	Trabzon	ISK3	40.93361	39.24477	749
Iskenderli	Trabzon	ISK4	40.93372	39.24592	778
Iskenderli	Trabzon	ISK5	40.9338	39.2507	808
Iskenderli	Trabzon	ISK6	40.93475	39.24976	788
Iskenderli	Trabzon	ISK7	40.93637	39.24982	807
Iskenderli	Trabzon	ISK8	40.93663	39.2477	747
Iskenderli	Trabzon	ISK9	40.9371	39.24781	767
Iskenderli	Trabzon	ISK10	40.93739	39.24738	777
Iskenderli	Trabzon	ISK11	40.93775	39.245	781

Table A.1 (Continued)

Region	City	Code	North	East	altitude
Iskenderli	Trabzon	ISK12	40.93811	39.24916	833
Iskenderli	Trabzon	ISK13	40.93792	39.25009	835
Iskenderli	Trabzon	ISK14	40.9376	39.25139	863
Iskenderli	Trabzon	ISK15	40.93901	39.25037	895
Iskenderli	Trabzon	ISK16	40.93834	39.2516	895
Iskenderli	Trabzon	ISK17	40.93809	39.25245	910
Iskenderli	Trabzon	ISK18	40.937	39.25286	917
Iskenderli	Trabzon	ISK19	40.93779	39.25338	939
Iskenderli	Trabzon	ISK20	40.93923	39.25338	988
Iskenderli	Trabzon	ISK21	40.93787	39.25427	988
Iskenderli	Trabzon	ISK22	40.93602	39.25489	995
Iskenderli	Trabzon	ISK23	40.93704	39.25465	1005
Hatila	Artvin	HAT1	41.146	41.6829	840
Hatila	Artvin	HAT2	41.13816	41.6549	1034
Hatila	Artvin	HAT3	41.13663	41.65244	1040
Hatila	Artvin	HAT4	41.12404	41.63536	1160
Hatila	Artvin	HAT5	41.12483	41.63546	1161
Hatila	Artvin	HAT6	41.12677	41.63668	1180
Hatila	Artvin	HAT7	41.12413	41.6347	1198
Hatila	Artvin	HAT8	41.12215	41.63476	1215
Hatila	Artvin	HAT9	41.1218	41.63126	1248
Hatila	Artvin	HAT10	41.12083	41.63163	1283

Table A.1 (Continued)

Region	City	Code	North	East	altitude
Hatila	Artvin	HAT11	41.12033	41.63292	1292
Hatila	Artvin	HAT12	41.11942	41.63396	1301
Hatila	Artvin	HAT13	41.12263	41.62646	1350
Hatila	Artvin	HAT14	41.12411	41.62856	1407
Hatila	Artvin	HAT15	41.12533	41.62376	1456
Hatila	Artvin	HAT16	41.12594	41.62044	1486
Hatila	Artvin	HAT17	41.12674	41.62368	1516
Hatila	Artvin	HAT17B	41.12737	41.62602	1539
Hatila	Artvin	HAT18	41.12784	41.62598	1570
Hatila	Artvin	HAT19	41.1275	41.62103	1617
Hatila	Artvin	HAT20	41.12874	41.61982	1637
Hatila	Artvin	HAT21	41.12747	41.61383	1716
Hatila	Artvin	HAT22	41.12723	41.60839	1782
Hatila	Artvin	HAT23	41.12741	41.60625	1803
Hatila	Artvin	HAT24	41.12873	41.60937	1833
Hatila	Artvin	HAT25	41.12985	41.61196	1853

APPENDIX B

CTAB METHOD (DOYLE AND DOYLE, 1990)

1. 0.1 g frozen leaf is put in a steril mortar and 1000 µL CTAB (Cetyl Trimethyl Ammonio Bromuro) buffer added.
2. The mixture is crushed until it takes a homogenous green color. Then, 700 µL CTAB and 200 µL β-mercapto-ethanol and 5 µl proteinase K is added to the mixture.
3. The mixture added eppendorf tubes are placed in 65 ºC water bath for 30 minutes.
4. After 30 minutes, eppendorf tubes are centrifugated at 4 ºC 15000 rpm for 15 minutes.
5. Supernatant part is transfered in a new tube and 500 µL phenol: chloroform: isoamyl alcohol (25:24:1) is added. Mixture is centrifugated again at 4 ºC 15000 rpm for 15 minutes in order to wash the supernatant.
6. Supernatant is transfered in a new tube and 500 µL chloroform IAA(24:1) is added and mixed slowly. Then it is centrifugated at 4 ºC 15000 rpm for 15 minutes.
7. Supernatant part is transfered in a new tube and 0,7 – 1 mL isoprophanol is added. DNA is visible as white color when the misture is shaken slowly.
8. Samples are kept at -20 ºC for 2 hours. Then, they are centrifugated at 4 ºC 13000 rpm for 8 minutes.
9. Pellet is washed with 500 µL %70 alcohol. Supernatant is removed and DNA is dried in an incubator at 30 ºC for 30 minutes.
10. After DNA is dry, 50 - 75 µL TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) is added and DNA is dissolved.
11. Stock DNA samples are stored at -80 ºC in freezer for future use.

APPENDIX C

PRIMER SEQUENCES, PRIMER SIZES AND DYES USED FOR EACH PRIMER

Table C.1

SSR locus	Applied species	Primer sequence (Forward, Reverse, 5'-3')	Motif	DYE	Annealing Temp.	Allele size (bp)	Source
WPMS 12	*Populus* sp.	TTTTTCGTATTCTTATCTATCC CACTACTCTGACAAAACCATC	$(GT)_{19}$	Tamra	55	152-235	Van Der Schoot *et al.* (2000)
WPMS 18	*Populus* sp.	CTTCACATAGGACATAGCAGCATC CACCAGAGTCATCACCAGTTATTG	$(GTG)_{13}$	Tamra	60	211-235	Smulders *et al.* (2001)
WPMS 14	*Populus* sp.	CAGCCGCAGCCACTGAGAAATC GCCTGCTGAGAAGACTGCCTTGAC	(CGT)	Hex	53	245	Smulders *et al.* (2001)
WPMS 15	*Populus* sp.	CAACAAACCATCAATGAAGAAGAC AGAGGGTGTTGGGGGTGACTA	(CCT)	Fam	54	193	Smulders *et al.* (2001)

Table C.1 (Continued)

SARE04	*Salix alba/excelsa*	GACTTCTAGTATTTCTACCCCCTC TATAATTGAGAAAGAAAAAAGACG	(AC)16C(AC)10	Fam	54	89-143	Lian *et al.* (2001)
SB24	*Salix alba/excelsa*	ACTTCAATCTCTCTGTATTCT CTATTTATGGGTTGGTCGATC	[TG]21AG[TG]3AG[TG]3 AG[TG]3AGTGAG[TG]3	Tamra	54	114- 281	Barker *et al.* (2003)
gSlMCT052	*Salix alba/excelsa*	ATTCTTTTTCCACTCGCCAC GGATTGACCCATCTCGATTC	(CT)15/(AG)20	Hex	58	272	Stamati *et al.* (2003)
SB196	*Salix alba/excelsa*	CTGTTTCCTGCCACTATTACC TATAATCTGTCTCCTTTTGGC	[GCC]9	Hex	54	169-184	Barker *et al.* (2003)
gSIMCT24	*Salix alba/excelsa*	TCATTTGCTCGATGAGGTTG GTGGTAGTTGCAAAAGGGGA	(CT)10	Fam	58	300	Stamati et al., (2003)
KARP_W293	*Populus* sp.	TGATTGGGCTAAAGATGAAGC AACTCAGCAACCACCAGAAAC	*	Fam	57	111-175	Barker et al., (2003)

APPENDIX D

A PART OF EXCEL MATRIX SHOWING GENOTYPES AS NUMBER VS ALLELE SIZES

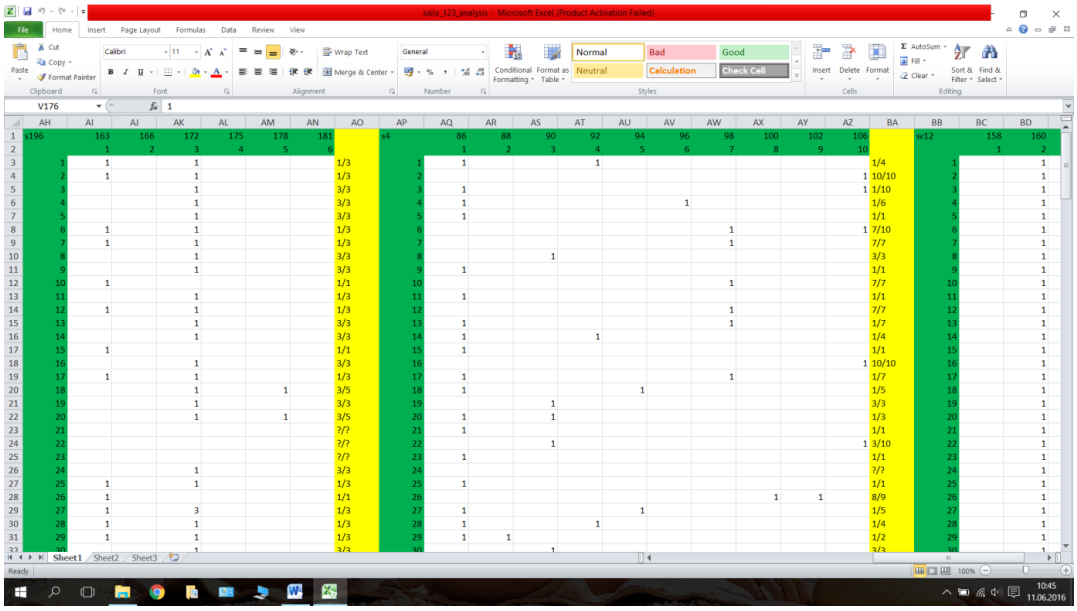


Figure D.1. Green colors at row represent alleles and green colors at columns represent individuals in the populations. Yellow colors represents alleles of individuals.

APPENDIX E

TABLE OF ESTIMATED NULL ALLELE FREQUENCIES

	0017 p	0008 p	0018 p	0027 p	0013 p	0021 p	0015 p	0014 p	0023 p	0024 p	**Mean**
WPMS14	0.0867	0.0000	0.0372	0.0000	0.1296	0.1024	0.0620	0.0000	0.1507	0.1435	**0.071**
WPMS15	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	**0.000**
SB24	0.0820	0.0000	0.0000	0.1976	0.0000	0.0550	0.1423	0.0105	0.1779	0.0241	**0.069**
SB196	0.0400	0.0000	0.0000	0.0335	0.0000	0.0000	0.0000	0.0000	0.0000	0.0018	**0.008**
Sare04	0.3327	0.0000	0.2179	0.2667	0.4359	0.1642	0.1305	0.2468	0.2230	0.1568	**0.217**
WPMS12	No inf	No inf	No inf	0.0000	0.0000	0.0000	0.2299	0.1541	0.0000	0.1017	**0.069**
WPMS18	0.2979	0.1641	0.0878	0.1955	0.2435	0.1460	0.0816	0.0000	0.0000	0.1036	**0.132**
GSLMCT052	0.1516	0.1641	0.0000	0.1807	0.0000	0.2719	0.2442	0.0935	0.0000	0.1620	**0.127**
GSIMCT24	0.0659	0.1456	0.0000	0.1364	0.2286	0.0739	0.2195	0.1237	0.1041	0.0688	**0.117**
KARP_W293	0.1255	0.0000	0.0394	0.0888	0.0828	0.2109	0.1927	0.2147	0.1122	0.0866	**0.115**

APPENDIX F

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 isoamil alcohol, (FLUKA) : (24/1)

Ethanol: 70% in distilled water

β mercapto ethanol, (SIGMA) : 17.5 ml β mercapto ethanol is completed with 250 ml with distilled water

TE buffer: 10mM 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: 10ng/ 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

Ethyidium Bromide: (SIGMA):4 mg/ ml

Agarose, (SIGMA): 3 % Agorose 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

Deepfreezer: 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.

APPENDIX G

A PART OF THE GDA DATA FILE

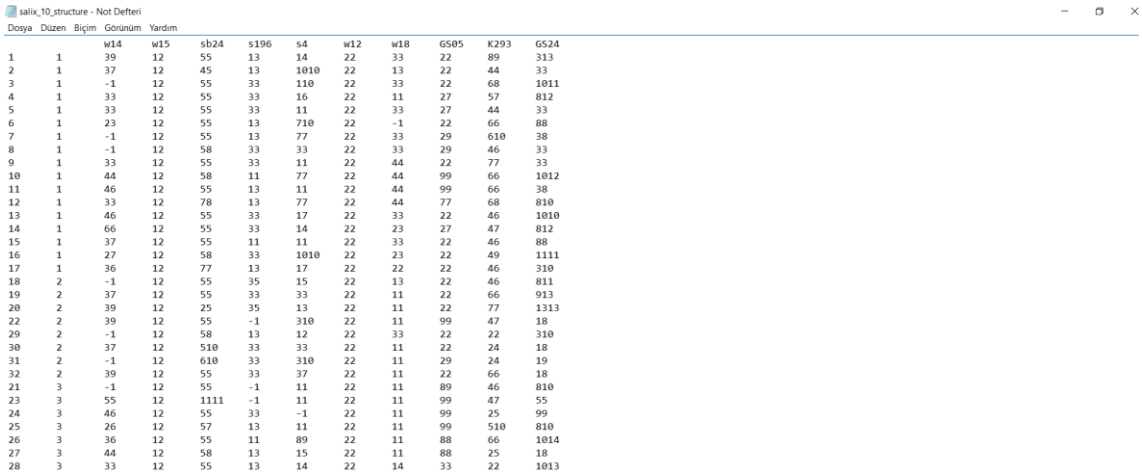
salix_10_gda - Not Defteri

Dosya Düzen Biçim Görünüm Yardım

```
#nexus

[!Data from Populus
]

begin gdadata;
     dimensions nloci=10 npops=10;
     format tokens missing=? datapoint=standard;
               locusallelelabels

               1 'WPMS14'   [/ 1 2 3 4 5 6 7 8 9],
               2 'WPMS15'   [/ 1 2 3 4],
               3 'SB24'   [/ 1 2 3 4 5 6 7 8 9 10 11],
               4 'SB196'   [/ 1 2 3 4 5 6],
               5 'Sare04'   [/ 1 2 3 4 5 6 7 8 9 10],
               6 'WPMS12'   [/ 1 2 3 4],
               7 'WPMS18'   [/ 1 2 3 4 5 6],
               8 'GS05'   [/ 1 2 3 4 5 6 7 8 9 10],
               9 'K293'   [/ 1 2 3 4 5 6 7 8 9 10],
               10 'GS24'   [/ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18];
matrix
eynesil:
1       3/9     1/2     5/5     1/3     1/4     2/2     3/3     2/2     8/9     3/13
2       3/7     1/2     4/5     1/3     10/10   2/2     1/3     2/2     4/4     3/3
3       ?/?     1/2     5/5     3/3     1/10    2/2     3/3     2/2     6/8     10/11
4       3/3     1/2     5/5     3/3     1/6     2/2     1/1     2/7     5/7     8/12
5       3/3     1/2     5/5     3/3     1/1     2/2     3/3     2/7     4/4     3/3
6       2/3     1/2     5/5     1/3     7/10    2/2     ?/?     2/2     6/6     8/8
7       ?/?     1/2     5/5     1/3     7/7     2/2     3/3     2/9     6/10    3/8
8       ?/?     1/2     5/8     3/3     3/3     2/2     3/3     2/9     4/6     3/3
9       3/3     1/2     5/5     3/3     1/1     2/2     4/4     2/2     7/7     3/3
10      4/4     1/2     5/8     1/1     7/7     2/2     4/4     9/9     6/6     10/12
11      4/6     1/2     5/5     1/3     1/1     2/2     4/4     9/9     6/6     3/8
12      3/3     1/2     7/8     1/3     7/7     2/2     4/4     7/7     6/8     8/10
13      4/6     1/2     5/5     3/3     1/7     2/2     3/3     2/2     4/6     10/10
14      6/6     1/2     5/5     3/3     1/4     2/2     2/3     2/7     4/7     8/12
15      3/7     1/2     5/5     1/1     1/1     2/2     3/3     2/2     4/6     8/8
16      2/7     1/2     5/8     3/3     10/10   2/2     2/3     2/2     4/9     11/11
17      3/6     1/2     7/7     1/3     1/7     2/2     2/2     2/2     4/6     3/10,
kafkasor:
18      ?/?     1/2     5/5     3/5     1/5     2/2     1/3     2/2     4/6     8/11
19      3/7     1/2     5/5     3/3     3/3     2/2     1/1     2/2     6/6     9/13
```

APPENDIX H

A PART OF THE STRUCTURE DATA FILE

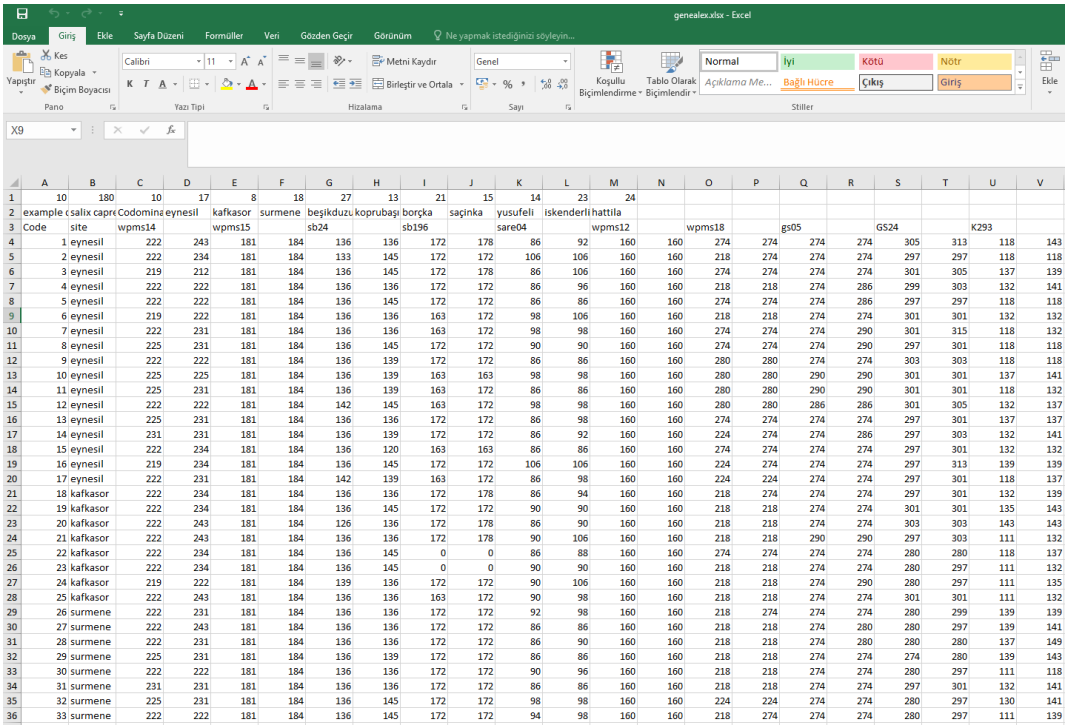

salix_10_structure - Not Defteri

Dosya Düzen Biçim Görünüm Yardım

		w14	w15	sb24	s196	s4	w12	w18	GS05	K293	GS24
1	1	39	12	55	13	14	22	33	22	89	313
2	1	37	12	45	13	1010	22	13	22	44	33
3	1	-1	12	55	33	110	22	33	22	68	1011
4	1	33	12	55	33	16	22	11	27	57	812
5	1	33	12	55	33	11	22	33	27	44	33
6	1	23	12	55	13	710	22	-1	22	66	88
7	1	-1	12	55	13	77	22	33	29	610	38
8	1	-1	12	58	33	33	22	33	29	46	33
9	1	33	12	55	33	11	22	44	22	77	33
10	1	44	12	58	11	77	22	44	99	66	1012
11	1	46	12	55	13	11	22	44	99	66	38
12	1	33	12	78	13	77	22	44	77	68	810
13	1	46	12	55	33	17	22	33	22	46	1010
14	1	66	12	55	33	14	22	23	27	47	812
15	1	37	12	55	11	11	22	33	22	46	88
16	1	27	12	58	33	1010	22	23	22	49	1111
17	1	36	12	77	13	17	22	22	22	46	310
18	2	-1	12	55	35	15	22	13	22	46	811
19	2	37	12	55	33	33	22	11	22	66	913
20	2	39	12	25	35	13	22	11	22	77	1313
22	2	39	12	55	-1	310	22	11	99	47	18
29	2	-1	12	58	13	12	22	33	22	22	310
30	2	37	12	510	33	33	22	11	22	24	18
31	2	-1	12	610	33	310	22	11	29	24	19
32	2	39	12	55	33	37	22	11	22	66	18
21	3	-1	12	55	-1	11	22	11	89	46	810
23	3	55	12	1111	-1	11	22	11	99	47	55
24	3	46	12	55	33	-1	22	11	99	25	99
25	3	26	12	57	13	11	22	11	99	510	810
26	3	36	12	55	11	89	22	11	88	66	1014
27	3	44	12	58	13	15	22	11	88	25	18
28	3	33	12	55	13	14	22	14	33	22	1013

APPENDIX I

A PART OF THE GENALEX DATA FILE



genealex.xlsx - Excel

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
1	10	180	10	17	8	18	27	13	21	15	14	23	24									
2	example (	salix capre	Codomina	eynesil	kafkasor	surmene	beşikduzu	koprubaşı	borçka	saçinka	yusufeli	iskenderli	hattila									
3	Code	site	wpms14		wpms15		sb24		sb196		sare04		wpms12		wpms18		gs05		GS24		K293	
4	1	eynesil	222	243	181	184	136	136	172	178	86	92	160	160	274	274	274	274	305	313	118	143
5	2	eynesil	222	234	181	184	133	145	172	172	106	106	160	160	218	274	274	274	297	297	118	118
6	3	eynesil	219	212	181	184	136	145	172	178	86	106	160	160	274	274	274	274	301	305	137	139
7	4	eynesil	222	222	181	184	136	136	172	172	86	96	160	160	218	218	274	286	299	303	132	141
8	5	eynesil	222	222	181	184	136	145	172	172	86	86	160	160	274	274	274	286	297	297	118	118
9	6	eynesil	219	222	181	184	136	136	163	172	98	106	160	160	218	218	274	274	301	301	132	132
10	7	eynesil	222	231	181	184	136	136	163	172	98	98	160	160	274	274	274	290	301	315	118	132
11	8	eynesil	225	231	181	184	136	145	172	172	90	90	160	160	274	274	274	290	297	301	118	118
12	9	eynesil	222	222	181	184	136	139	172	172	86	86	160	160	280	280	274	274	303	303	118	118
13	10	eynesil	225	225	181	184	136	139	163	163	98	98	160	160	280	280	290	290	301	301	137	141
14	11	eynesil	225	231	181	184	136	139	163	172	86	86	160	160	280	280	290	290	301	301	118	132
15	12	eynesil	222	222	181	184	142	145	163	172	98	98	160	160	280	280	286	286	301	305	132	137
16	13	eynesil	225	231	181	184	136	136	172	172	86	98	160	160	274	274	274	274	297	301	137	137
17	14	eynesil	231	231	181	184	136	139	172	172	86	92	160	160	224	274	274	286	297	303	132	141
18	15	eynesil	222	234	181	184	136	120	163	163	86	86	160	160	274	274	274	274	297	301	132	132
19	16	eynesil	219	234	181	184	136	145	172	172	106	106	160	160	224	274	274	274	297	313	139	139
20	17	eynesil	222	231	181	184	142	139	163	172	86	98	160	160	224	224	274	274	297	301	118	137
21	18	kafkasor	222	234	181	184	136	136	172	178	86	94	160	160	218	274	274	274	297	301	132	139
22	19	kafkasor	222	234	181	184	136	145	172	172	90	90	160	160	218	218	274	274	301	301	135	143
23	20	kafkasor	222	243	181	184	126	136	172	178	86	90	160	160	218	218	274	274	303	303	143	143
24	21	kafkasor	222	243	181	184	136	136	172	178	90	106	160	160	218	218	290	290	297	303	111	132
25	22	kafkasor	222	234	181	184	136	145	0	0	86	88	160	160	274	274	274	274	280	280	118	137
26	23	kafkasor	222	234	181	184	136	145	0	0	90	90	160	160	218	218	274	274	280	297	111	132
27	24	kafkasor	219	222	181	184	139	136	172	172	90	106	160	160	218	218	274	290	280	297	111	135
28	25	kafkasor	222	243	181	184	136	136	163	172	90	98	160	160	218	218	274	274	301	301	111	132
29	26	surmene	222	231	181	184	136	136	172	172	92	98	160	160	218	274	274	274	280	299	139	139
30	27	surmene	222	243	181	184	136	136	172	172	86	86	160	160	218	218	274	280	280	297	139	141
31	28	surmene	222	231	181	184	136	136	172	172	86	90	160	160	218	218	274	280	280	280	137	149
32	29	surmene	225	231	181	184	136	139	172	172	86	86	160	160	218	218	274	274	274	280	139	143
33	30	surmene	222	222	181	184	136	136	172	172	90	96	160	160	218	218	274	274	280	297	111	118
34	31	surmene	231	231	181	184	136	136	172	172	86	86	160	160	218	218	274	274	297	301	132	141
35	32	surmene	225	231	181	184	136	145	172	172	98	98	160	160	224	224	274	274	280	297	130	141
36	33	surmene	222	222	181	184	136	145	172	172	94	98	160	160	218	274	274	274	280	297	111	139