DOI: 10.5152/forestist.2020.19029
Forestist 2020, 70(2): 141-150

İSTANBUL UNIVERSITY
CERRAHPAŞA

Original Article

Turkish *Salix* species: Molecular phylogeny and morphology

Türkiye *Salix* türleri: Moleküler filogeni ve morfoloji

Pelin Acar[1], Bircan Taşkıran[2], Funda Ö. Değirmenci[2,3], Zeki Kaya[2]

[1]National Botanical Garden of Turkey, Ministry of Agriculture and Forestry, Ankara, Turkey
[2]Department of Biological Sciences, Middle East Technical University, Ankara, Turkey
[3]Department of Field Crops, Ahi Evran University Faculty of Agriculture, Kırşehir, Turkey

ABSTRACT

This study provides a new insight into Turkish *Salix* L. systematics, using a molecular phylogeny and numerical morphometric analysis approach. Despite its economic importance for bioenergy, there is to date no record of any extensive study on this Turkish willow species. Twenty-four *Salix* species and one hybrid were subjected to molecular and morphometric evaluation, in which one gene region of the external transcribed spacer (*ETS*) of the 18S-26S nuclear ribosomal DNA and 11 morphological characters were analyzed using a Bayesian Analysis of Beast program and Multiple Correspondence Analysis (MCA) in R. The results indicate that *Salix* species in Turkey could be accurately classified at the subgenera level, considering the selected gene region and morphological traits (subgenus *Salix* and *Vetrix*). Life form, leaf shape (*Dim 1*) and bud scale (*Dim 3*) were highly discriminative at the subgenera level. The molecular and morphological data confirmed that the taxonomic position of *Salix amplexicaulis* needs to be changed as subgenus *Salix*. Additionally, the members of subgenus *Salix*, *S. acmophylla* and *S. pentandroides* were all clustered distantly from other species of the subgenus.

Keywords: Turkish willows, phylogeny, nrDNA, external transcribed spacer, numerical taxonomy

ÖZ

Bu çalışma, Türk *Salix* L. sistematiğine moleküler filogenetik ve sayısal morfometrik analiz yaklaşımı kullanarak yeni bir bakış açısı sunmaktadır. Biyoenerjide ekonomik açıdan önemli olmasına rağmen, Türkiye Söğüt türlerinde bu güne kadar kapsamlı bir çalışma bulunmamaktadır. Çalışmada 18S-26S çekirdek ribosomal DNA 'Eksternal transcribed spacer' (*ETS*) gen bölgesi ve on bir bilgilendirci morfolojik karakter seçilerek, sırasıyla Beast programı, Bayesian ve R paketi, Multiple Correspondence Analizleri (MCA) ile yirmi dört *Salix* türü ve bir melezde değerlendirme yapılmıştır. Sonuçlar ışığında Türkiye'deki *Salix* türleri, seçilen gen bölgesi ve morfolojik özelliklere göre altcins seviyesinde düzgün bir şekilde ayrılmaktadır (Altcins *Salix* ve *Vetrix*). Hayat formu, yaprak şekli (*Dim1*) ve tomurcuk pulu (*Dim3*) altcins düzeyinde oldukça ayırt edici karakterlerdir. Moleküler ve morfolojik veriye göre *Salix amplexicaulis* türünün taksonomik pozisyonu altcins *Salix* olarak değiştirilmelidir. Ayrıca, altcins *Salix* üyelerinden *S. acmophylla* ve *S.pentandroides* altcinsin diğer türlerinden her zaman uzakta konumlanmaktadır.

Anahtar Kelimeler: Türkiye söğütleri, filogeni, nrDNA, external transcribed spacer, numerik taksonomi

Cite this paper as:
Acar, P., Taşkıran, B., Değirmenci, F.Ö., Kaya, Z., 2020. Turkish Salix species: Molecular phylogeny and morphology. *Forestist* 70(2): 141-150.

Corresponding author:
Pelin Acar
e-mail:
pelinkeske@gmail.com

Received Date:
18.09.2019
Accepted Date:
09.12.2019

INTRODUCTION

With over 500 species globally, *Salix* L. is the largest genus of the Salicaceae (Argus, 1997), occurring mainly in the Northern Hemisphere. There are 65 species in Europe (Kuzovkina and Quigley, 2005) and 27 species in Turkey (Terzioğlu et al., 2014). Four of these 27 *Salix* species are endemic to Turkey, including *S. trabzonica* A. Skv., *S. purpurea* subsp. *leucodermis* L., *S. rizeensis* A. Güner et al. J. Zielinski and *S. anatolica* J. Zielinski and D. Tomaszewski (Güner, 2000; Zielinski and Tomaszewski, 2007). The phytogeographical distributions of some Turkish *Salix* species correspond to the geographical regions where they are naturally found. For example, *Salix aegyptiaca* L. (Iran-Turan element) is naturally found in the Southeast Anatolia Region (Avcı, 1999). The richest region of Turkey for *Salix* L. spe-

cies (23 species) is the Black Sea Region, followed by the Eastern Anatolia Region with 15 species. The region with the least number of *Salix* L. (6 species) is the Southeast Anatolia Region (Arıhan and Güvenç, 2011). As members of the *Salix* genus have small seeds suited for wind dispersion, they can colonize diverse habitats ranging from arid areas to wetlands, from beaches to high mountains (Skvortsov, 1999). In general, systematics data for angiosperms are mainly derived from flower-based characteristics. However, important floral characteristics used in taxonomic studies are absent in *Salix* species (Azuma et al., 2000), as *Salix* sp. only has reduced flowers over a very short period in the spring. Therefore, only vegetative traits can be used in *Salix* systematics, as demonstrated in this study. There are numerous systematic studies on *Salix* based on morphological traits, which require careful evaluation, as the infrageneric classification of *Salix* depends on different authors' treatments. Skvortsov (1999) reviewed Turkish *Salix* species listed in Davis (1965-1988) and reported the existence of 2 subgenera (*Salix* and *Vetrix*) with 13 sections. Despite another recently published paper (Degirmenci et al., 2019), this plant genus is one of the most poorly understood in Turkey.

Economically, *Salix* species are excellent candidates for bioenergy production (Vermerris, 2008). Some clones of *Salix* species are used in forest biotechnology for their characteristic quick growth, wide distribution, and resistance to disease and stress (Herrera, 2006). Shrub willows, in particular, have shown to be reliable bioenergy crops, due to their high growth and yield rate in forestry. Willow plantations also mitigate erosion and have a significant impact on afforestation. However, the number of studied willow clones are limited in Turkey (Akgul and Tuctaner, 2011).

The existence of speciation forces within the *Salix* genus, such as introgressive hybridization, often leads to reticulate taxonomical relations (Azuma et al., 2000; Suda and Argus, 1968). With the increasing problem of uniparental inheritance in phylogeny, rather than cpDNA regions, a number of studies have been conducted on nuclear sequence markers in plant systematics to solve this complex relation. Recently, the nuclear ribosomal DNA *ETS* gene region has been extensively studied in molecular phylogenetic, due to its high polymorphism rate (Weeks et al., 2004). Although *ETS* is a short gene region, it was found that *ETS* sequence data is unique in *Salix* species (Wu et al., 2015). As traditional methods to identify Salicaceae species using only morphological traits are not sufficient to classify them (due to hybridization, reproductive isolation, and polyploidy), the external transcribed spacer (*ETS*) of the 18S-26S nuclear ribosomal DNA was sequenced in 26 representative taxa of the *Salix* L. genus in Turkey. The combination

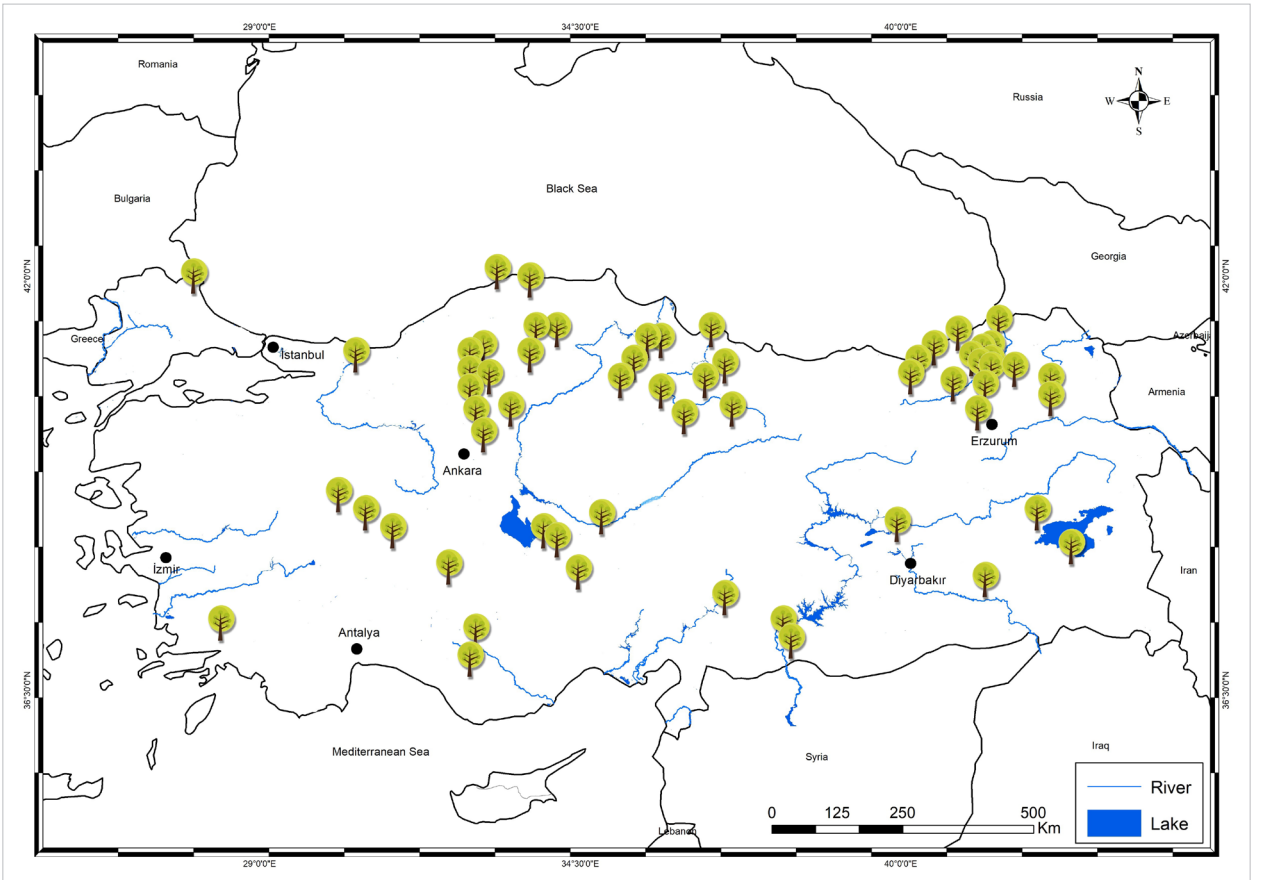

Figure 1. The locations of sampled Turkish *Salix* L. species

of molecular sequences and morphometric data based on an appropriate vegetative character set allowed scientists to be familiar to this genus. In this study, the infrageneric problems of 24 Turkish *Salix* species and one hybrid were studied using molecular and morphometric analysis to bring new insight in *Salix* taxonomy.

Table 1. The list of *Salix* species, given codes, and the number of samples representing each species and their location

The code	Species	Subgenus	# Morphological Samples used	District Name/ Province
ALBA	*Salix alba*	*Salix*	55	Akyazı-Vakıf/Sakarya, Çatak/Konya, Beynam/Ankara, Çoruh/Artvin, İspir/Erzurum, Bor/Niğde, Ürgüp/Nevşehir, Uluırmak Köprüsü/ Aksaray
EXCE	*S. excelsa*	*Salix*	41	Çelikli/Samsun Kışlacık Köyü/Kırklareli, Ovacık Köyü/Sivas, Yusufeli/Artvin
TRIA subsp tri	*S. triandra* subsp. *triandra*	*Salix*	29	Çerkeş Orman Fidanlığı/Çankırı, Üçköy/Çorum, Tokat, Afyon, İspir/Erzurum, Tosya-Beşçam/Kastamonu, Ihlara Vadisi /Aksaray
TRIA subsp bor	*S. triandra* subsp. *bornmuelleri*	*Salix*	2	Çeltek-Tersakan/Amasya
BABY	*S. babylonica*	*Salix*	22	Yaylacık Köyü/Amasya Tokat, ÇoruhYaylacık Çıkışı/Artvin, Kalecik/Ankara, Ihlara vadisi/Aksaray
PENT	*S. pentandroides*	*Salix*	6	Topulyurdu/Sivas, Beynam/Ankara, Çarşamba/Samsun, Çoruh-Bağbaşı/Erzurum, Güleman-Ayıpınar/Elazığ, Ladik Amasya
ALBxfra**	*S. alba x fragilis*	*Salix*	1	Beynam/Ankara
ACMO	*S. acmophylla*	*Salix*	2	Asma Köprü Suçeken/Batman, Birecik /Şanlıurfa
FRAG	*S. fragilis*	*Salix*	12	Çay/Afyon, BeynamOrmanı /Ankara, Akşehir/Konya
CINE	*S. cinerea*	*Vetrix*	11	Akyazı Gebeş/Sakarya, Çubuk-Karagöl/ Ankara, Çoruh Bağbaşı / Erzurum
PSEUDO	*S. pseudomedemii*	*Vetrix*	2	Zile/Tokat, Beynam/ Ankara
AEGY	*S. aegyptiaca*	*Vetrix*	2	Kars-Erzurum Yolu /Erzurum, Bahçesaray /Van
WILH	*S. wilhelmsiana*	*Vetrix*	3	Kars-Erzurum Yolu /Erzurum, İkizdere/Rize
VIMI	*S. viminalis*	*Vetrix*	1	Nehir Başı/Erzurum
PEDI subsp pe	*S. pedicellata* subsp. *pedicellata*	*Vetrix*	3	Göksu-Ermenek/Karaman, Maraş
AMPL	*S. amplexicaulis*	*Vetrix*	3	Çubuk-Kızılcahamam /Ankara, Ilgaz/Kastamonu
ELBU	*S. elbursensis*	*Vetrix*	3	Çoruh-Alanbaşı/Artvin
ARME	*S. armenorossica*	*Vetrix*	2	Bağbaşı-Çoruh/Erzurum
ELAE	*S. elaeagnos*	*Vetrix*	3	Ilgaz/ Kastamonu
CAPR	*S. caprea*	*Vetrix*	3	Kızılcahamam/ Ankara, Kastamonu-Çankırı il sınırı, Kafkasör Yaylası/Artvin, Bostan/Kastamonu
CAUC	*S. caucasica*	*Vetrix*	3	Ayder/Rize, Çoruh-Sırakonaklar/Artvin
APOD	*S. apoda*	*Vetrix*	1	Ladik/ Amasya
PURP subsp leu	*S. purpurea* subsp. *leucodermis*	*Vetrix*	1	Köyceğiz/ Muğla
MYRS	*S. myrsinifolia*	*Vetrix*	1	Ilgaz/Kastamonu
RIZE*	*S. rizeensis* (23.08.1985/A.Guner-M.Vural / HUB 06442)	*Vetrix*	1	İkizdere/Rize
PSEUDEP*	*S. pseudodepressa* (1981/A.Guner/ HUB 06440)	*Vetrix*	1	Gümüş Damla Köyü/Bayburt

*Herbarium species with voucher information
**Hybrid species

MATERIALS AND METHODS

Study Materials

In total, 214 samples of 26 *Salix* taxa (including one hybrid) from different regions of Turkey were collected and identified (Figure 1). Among these, 45 samples were used to generate molecular data. The codes, sample sizes and locations of each species are provided in Table 1. The topographic and geographic information of samples were provided in more detail in Acar (2017). The duration of field studies for collecting fresh shoots and leaves were limited to the spring and early summer. In the field, shoots with fresh leaves were preserved in packages with silica gel for molecular analyses and pressed for morphological analyses. Herbarium samples of *S. pseudodepressa* A. Skv. and *S. rizeensis* from the Hacettepe University Herbarium (HUB) were also analyzed. Unfortunately, the endemic species *S.trabzonica* and *S.anatolica* could not be obtained, although field trips were done to record habitats and herbariums were also checked. *Populus cathayana* was used as an outgroup in our phylogenetic tree. The specimens were identified using the Flora of Turkey and the East Aegean Islands, Vol. 7 (Davis 1965, 1988). Identification issues were resolved by consulting the book by Skvortsov (1999).

Data Collection and Analysis

Nuclear DNA was isolated using the modified Cetyl Trimethyl Ammonium Bromide method from the leaves (Doyle and Doyle 1987). DNA presence and quality were checked and diluted DNA samples (10 ng/µL) were stored at 4^{0}C for a short period. Nuclear ribosomal *ETS* (Baldwin and Markos, 1998) gene regions were amplified and sequenced using universal primers (at least one sample for each *Salix* species). PCR amplification was accomplished in 20 µL reactions using the 5X HOT FIREPol Blend PCR Mix (with 15Mm $MgCl_2$; Solis Byodyne, Estonia). PCR reactions were performed with: 3 µL PCR Mix, 0.5 µL each primer pair, 4 µL template DNA and 12 µL water in 0.2 mL sterile Eppendorf tubes. The reactions were performed as initial denaturation at 95°C for 5 min followed by of 1 min at 94°C, 1 min at 58°C for annealing, 2 min at 72°C; and followed by a final extension at 72°C for 10 min. Agarose gels in 1% and 1.5% concentrations were used to run PCR samples. The purification and sequencing procedures were performed by the Genoks Molecular Biotechnology Company (Cinnah, Ankara), a European BGI agent. An ABI3730XL 96 capillary automatic sequencer was used for the sequencing of amplified DNA products. The multiple alignment was done using the CLUSTAL W software and Finch TV (Version 1.4.0) developed by the Geopiza Research Team, to view the chromatogram data and to check base positions (Patterson et al., 2004-2006). Molecular parameters were estimated with the MEGA 6.0 software (Tamura et al., 2013). A phylogenetic tree was constructed based on maximum parsimony, maximum likelihood, and Bayesian inference. DnaSP v5 (Librado and Rozas, 2009) was used to get a nexus format file, which was uploaded to BEAUti software to get an eXtensible Markup Language (XML) file. The phylogenetic tree was created using BEAST version 1.8.4 (Drummond and Rambaut, 2007) under a coalescent tree prior and random starting tree model

Table 2. List of studied morphological characters and their respective units

Number	Character	Scoring of traits	Units
1	Life form (Lf)	Tree or not	Binary; yes=1, no= 0
2	Bud scale (Bs)	Glabrous or not	Binary; yes=1, no= 0
3	Brunch habit (Bh)	Dropping or not	Binary; yes=1, no= 0
4	Bark type (Bt)	Fissured or smooth	Binary; yes=1, no= 0
5	Stipule persistence (Sp)	Persist or not	Binary; yes=1, no= 0
6	Decorticated wood (Dw)	Smooth or not	Binary; yes=1, no= 0
7	Leaf shape (Ls)	Lanceolate or not	Binary; yes=1, no= 0
8	Leaf color (Lc)	Dark green above or not	Binary; yes=1, no= 0
9	Twig slender (St)	Slender or not	Binary; yes=1, no= 0
10	Bud angle (Ba)	Angle btw bud and stem (degree)	1=0-10, 2=10.01-20,
			3=20.01-30, 4=30.01-40,
			5=40.01-50, 6=50.01-60,
			7=60.01-70
11	Petiole length (Pl)	Length (mm)	1=0.5-1.49, 2=1.5-2.49,
			3=2.5-3.49, 4=3.5-4.49,
			5=4.5-5.49, 6=5.5-6.49,
			7=6.5-7.49, 8=7.5-8.49,
			9=8.5-9.49

for each partition with four gamma categories, after running it for 10 million generations of the Markov Chain Monte Carlo. Since there is no intraspecific differentiation according to the selected gene region, only one taxon was used to represent one species in the tree. The software Tree Annotator v1.7.5 was used to estimate the maximum-clade-credibility using the Bayesian posterior probability showing the node base statistic. The tree was visualized in the Fig Tree v1.4.3 software (Rambaut, 2016).

Morphological characteristics were identified for inclusion into the morphological dataset. Some of these traits were selectively eliminated based on their non-discriminative features in the *Salix* genus by consulting the Flora of Turkey (Davis, 1965-1988). As it was difficult to obtain generative parts of the samples, particularly in the herbarium samples, only discriminative vegetative traits were included the final dataset. Morphometric measurements were made in the field, using fresh and herbarium samples, using a Leica MZ16 Fluorescence Stereomicroscope and Leica microscope camera. The data matrix was formed with nine morphological characters belonging to *Salix* taxa was standardized with binary coding (Table 2). Two more continuous characters: bud angle (Ba) and petiole length (Pl) were generated by measuring characters on photographs processed with a stereomicroscope (Figure 2). Petiole length was measured using three leaves for each individual species, using the average value of the three measurements. These continuous variables were converted to categorical nominal variables using IBM SPSS Statistic (22.0) for Multiple Correspondence Analysis (MCA). MCA is an extension of correspondence analysis which allows the analysis of relationship patterns of several categorical dependent variables (Abdi and Valentin, 2007). Technically, MCA is obtained by using a standard correspondence analysis on an indicator matrix (i.e., a matrix with binary entries). This statistical technique aims to extract important information from the dataset and provides this information as relationships between categorical dependent variables. A morphometric numerical analysis with 11 morphological characters for Turkish *Salix* genus was carried out with a Multiple Correspondence Analysis (MCA) using the R function "mca" of "FactoMinerR" package (R Core Team, 2014).

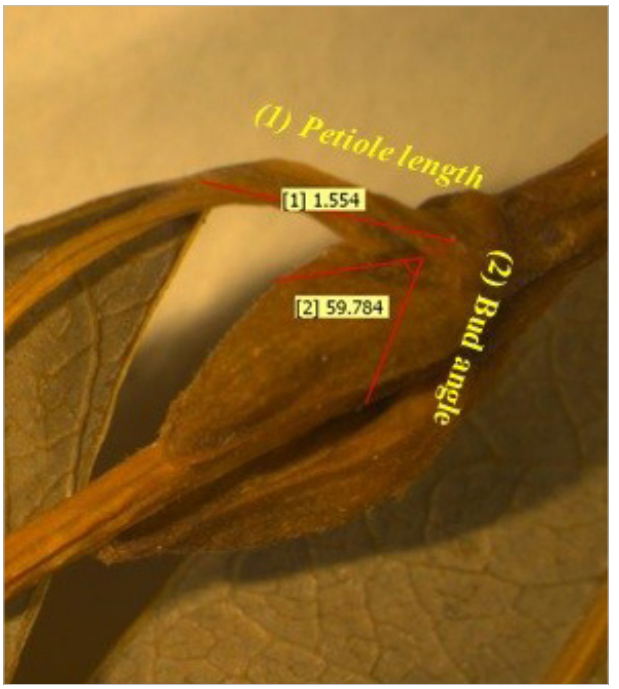

Figure 2. *Salix purpurea* subsp. *leucodermis* leaf image including Petiole length /Pl (1) and Bud angle/ Ba (2) generated using a Leica MZ16 Fluorescence Stereomicroscope and taken by a Digital Firewire Color Camera System (Leica DFC320)

RESULTS AND DISCUSSION

Molecular Analysis

The total length of rDNA *ETS* was 346 bp (Table 3). Polymorphism levels were high in the *ETS* gene region of the *Salix* species, at 14/346. All variable sites were informative. The measure of polymorphism of the overall sequences and nucleotide diversity was as high as 0.020. A high level of GC was observed, which is an indicator of high genomic variation in the DNA sequence. Therefore, this suggests that the *ETS* gene region was quite diverse and characteristically unique for the Turkish *Salix* species. Twelve variable sites in the *ETS* sequence were responsible for the divergence of subgenera of Turkish *Salix* species at 90, 106, 108, 158, 182, 194, 224, 262, 265, 278, 288, and 292th base positions (Table 4). There is no indel (insertion/deletion) for the selected gene region, showing that this is an important function of this region in evolution and conservation of *Salix* species. The phylogenetic tree constructed with sequence data from the *ETS* gene region supported two major groups (subgenera *Salix* and *Vetrix)* with high posterior probability values (Figure 3). Our results from ribosomal nuclear DNA data supported the classification system of Skvortsov (1999) in which Turkish *Salix* L. species can be grouped into two subgenera (*Salix* and *Vetrix*). Similar clade formations were also reported for Japanese (Azuma et al.,

Table 3. Estimated molecular diversity parameters based on the nuclear ribosomal DNA *ETS* gene region of Turkish *Salix* species

	nrDNA
	ETS (external transcribed spacer)
Number of species	24+1 hybrid*
Number of total sequences	45
Total length (basepairs)	346
GC content (%)	59.6
Conserved sites	332
Variable sites	14
Parsimony informative sites	14
Number of indels (insertion and deletion)	0
Nucleotide diversity	0.020

*S.*alba x fragilis* as hybrid species.

Table 4. Substitution positions in the nrDNA sequence representing the discrimination of two subgenera and the divergence positions of four *Salix* species

nrDNA ETS (nuclear DNA external transcribed spacer)	*Position of base*	*Subgenus Salix*	*Subgenus Vetrix*	*S. amplexicaulis*	*S. rizeensis*	*S. pentandroides*	*S. acmophylla*
	90	C	T	C	C	C	C
	106	C	T	C	T	C	T
	118	G	A	G	A	G	G
	158	T	C	T	T	T	T
	182	T	C	T	T	T	T
	194	T	C	T	T	T	T
	224	C	G	C	G	C	G
	262	A	C	A	A	C	A
	265	C	T	C	T	C	C
	278	A	G	A	A	A	A
	288	G	A	G	G	G	G
	292	A	G	A	A	A	A

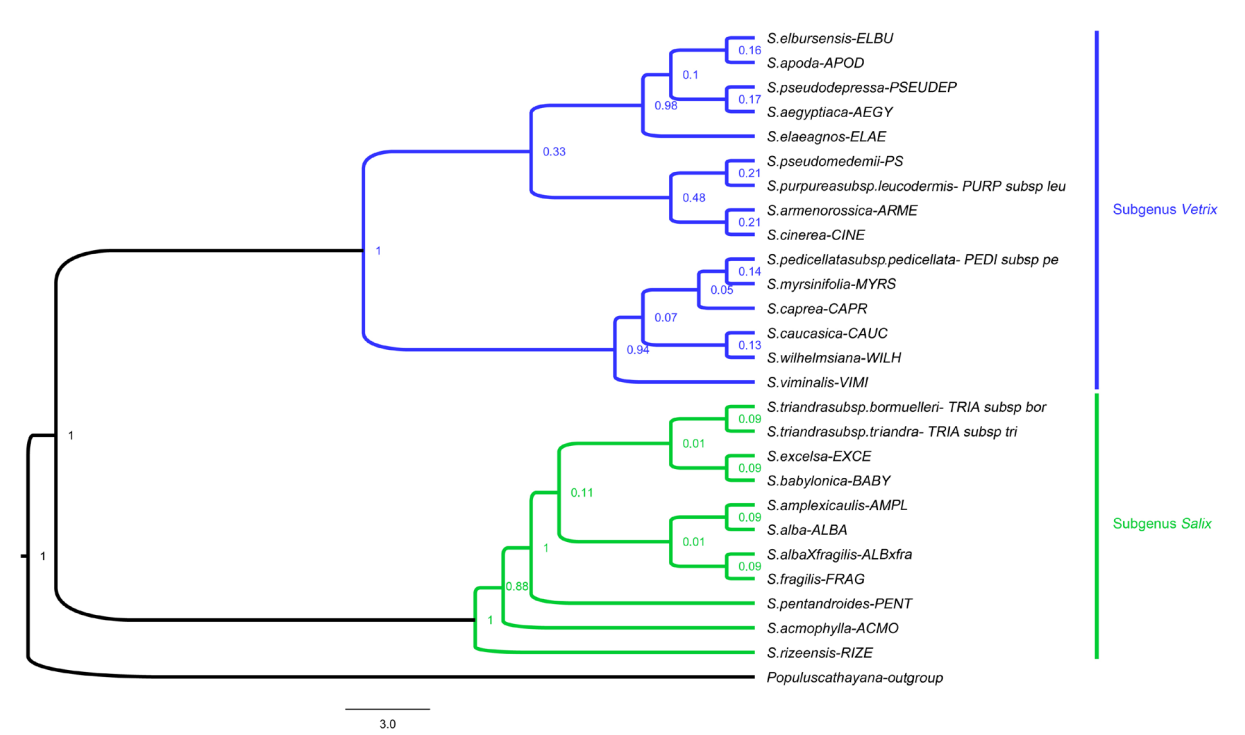

Figure 3. Best nuclear ribosomal DNA *ETS* gene tree for Turkish *Salix* sp.

2000), Chinese (Chen et al., 2010) and American *Salix* sp. (Lauren-Moreau et al., 2015). The first group of constructed Beast *ETS* tree was the subgenus *Vetrix* group, which had four subclades with low posterior values. The first subclade diverging had a high posterior value (0.98) consisting of *S.elbursensis* Boiss.-*S. apoda* Trautv, *S.pseudodepressa-S.aegyptiaca* pairs and *S.elaeagnos* Scop. which attach to pairs externally. The second subclade involves the pairs *S.pseudomedemii* E. Wolf -*S.purpurea* subsp. *leucodermis*, *S.armenorossica* A. Skv. -*S.cinerea* L. Additionally, *S.caprea* L. attached to the pair, *S.pedicellata* subsp. *pedicellata* Desf.-*S. myrsinifolia* in the third subclade. The fourth subclade was made up of one pair: *S.caucasica* Andersson and *S.wilhelmsiana* Bieb. The species *S.viminalis* L. was attached from outside to all species of the third and fourth subclades with high posterior

values of 0.94. In the second group, the subgenus *Salix* included four subclade pairs, *S.triandra* subsp. *triandra* L. - *S.triandra* subsp. *bornmuelleri* (Hausskn.) A. Skv., *S.excelsa* J.F. Gmelin-*S.babylonica* L., *S.amplexicaulis* Bory and Chaub - *S.alba* L., and *S.alba* x *fragilis*-*S.fragilis* L. with the same posterior number of 0.09. Two subspecies of *S.triandra* were placed at the upper position among subg. *Salix* species. Reticulated and complex relationships were found in subg. *Vetrix*, while closely relationships observed in subg. *Salix* members. The extensive polytomy of subg. *Vetrix* was reported in previous studies (Abdollahzadeh et al., 2011; Barkalov and Kozyrenko, 2014). Variable sites with complex relations had a higher detection rate in subg. *Vetrix* than in subg. *Salix* for this gene region. The results of the substitutions at the 90, 106, 118, 158, 182, 194, 224, 262, 265, 278, 288, and 292th bp positions of *S. amplexicaulis*, and at the 90, 106, 158, 182, 194, 262, 278, 288, and 292th bp positions of *S. rizeensis* were clustered along with the subgenus *Salix* rather than clustering with members of the subgenus *Vetrix*. The appearance of the subg. *Vetrix* members, *S. amplexicaulis* and *S. rizeensis* in the subg. *Salix* group can be explained by the natural hybridization occurring in mixed habitats. Furthermore, the subgenus *Salix* members, *S. acmophylla* Boiss. (106 and 224th bp) and *S. pentandroides* A. Skv. (262th bp) placed outside in this group as a result of the substitutions (Table 4).

Morphometric Analysis

Our MCA results indicate that different sets of characters are informative for clustering *Salix* taxa in two dimensions (Figure 4). Based on their morphological characters, a two-dimensional configuration of the MCA revealed two major clusters (subg. *Salix* and *Vetrix*) in the analysis (Figure 5). The subg. *Salix* samples were widely distributed and very accessible compared to the subg. *Vetrix*, which includes all endemic *Salix* species in Turkey. The first three dimensions explained 33.3% of the total morphometric variation. The first axis (*Dim1*) explained 16.9%, the second axis (*Dim2*) 9.2 % and the third axis (*Dim3*) 7.2% of the total variation. Thus, for the MCA analysis, a two-dimensional MCA solution was considered as the most satisfactory. Considering variables in *Dim1*, it is clear that life form, bark type, stipule persistence, leaf shape and twig slender had high loading scores. This suggests that these traits are important in the differentiation of species by *Dim1* (Table 5). Four traits with high loadings in *Dim2* were brunch habit, decorticated wood, bud angle and petiole length, which are also important features in *Salix* species classification. All discriminant measures were below 0.76, with a maximum value of 0.752 (leaf shape/Ls) for the first dimension (*Dim1*) and 0.578 (decorticated wood/Dw) for the second dimension (*Dim2*) (Table 5).

The cluster formations in Figure 4 show that *S. babylonica* (cluster 1), *S. triandra* subsp. *triandra* (cluster 2), *S. excelsa* (cluster 3), *S. fragilis* (cluster 4) and *S. alba* (cluster 5), belonging to subgenus *Salix* were clearly separated by *Dim1*. Although there were a few individuals which were outside the species' clusters, the majority of individuals showed consistency in species clustering. In particular,

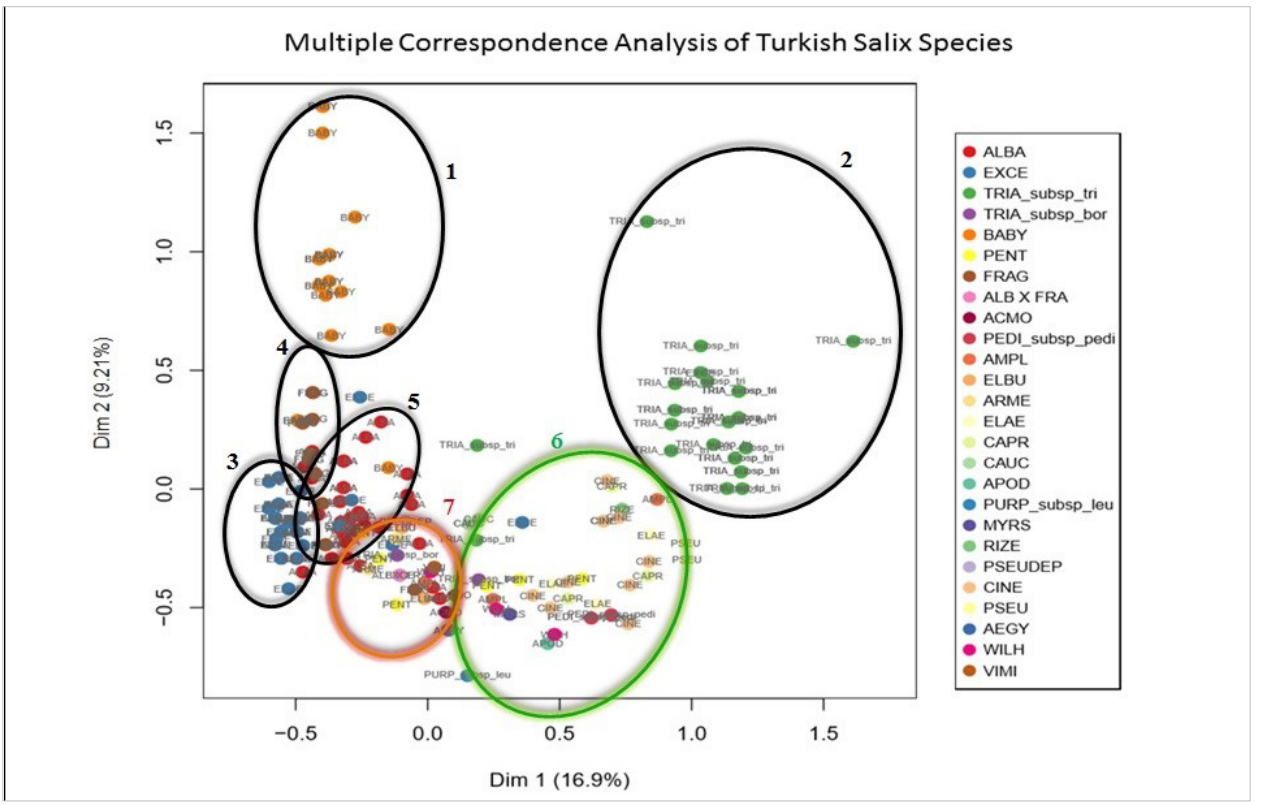

Figure 4. Plot of the MCA analysis with Turkish *Salix* L. taxa, indicating the clustering patterns revealed by the first two dimensions (*Dim1* and *Dim2*)

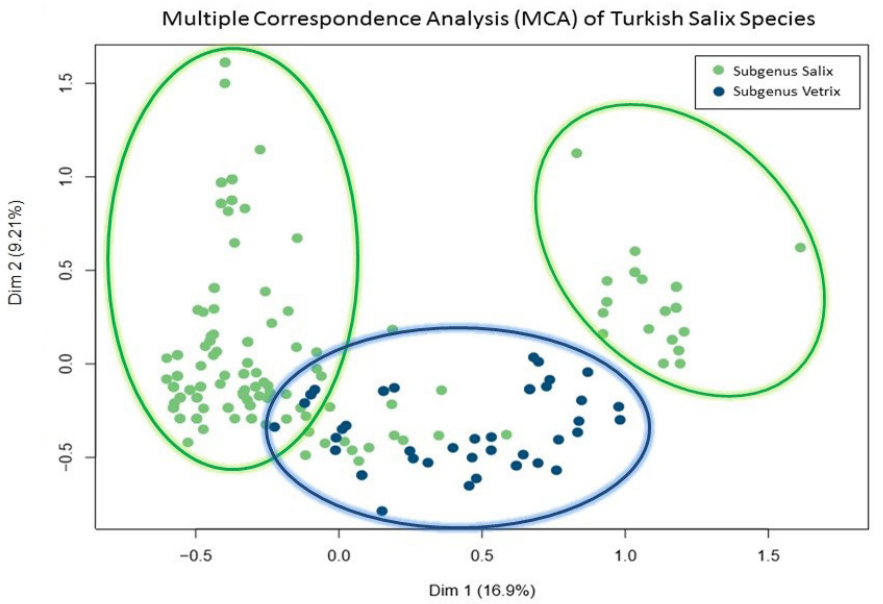

Figure 5. Plot of the MCA analysis with Turkish *Salix* L. subgenera (*Salix* and *Vetrix*), indicating the clustering patterns revealed by the first two dimensions (*Dim1* and *Dim2*)

Table 5. Summary of characteristics with the highest loadings (*) on the first three dimensions of MCA

Number	Character	*Dim1* (first axis)	*Dim2* (second axis)	*Dim3* (third axis)
1	Lf (Life form)	0.660*	0.010	0.004
2	Bs (Bud scale)	0.066	0.018	0.260*
3	Bh (Brunch habit)	0.046	0.505 *	0.148
4	Bt (Bark type)	0.576*	0.022	0.042
5	Sp (Stipule persistence)	0.416*	0.004	0.024
6	Dw (Decorticated wood)	0.136	0.578*	0.008
7	Ls (Leaf shape)	0.752*	0.000	0.000
8	Lc (Leaf color)	0.263	0.087	0.155
9	St (Twig slender)	0.651 *	0.027	0.072
10	Ba (Bud angle)	0.090	0.313*	0.381*
11	Pl (Petiole length)	0.062	0.463*	0.490*

all representatives of the exotic species *S. babylonica* was grouped into cluster 1 (based on *Dim2*) to which brunch habit contributed the most. Over 20 samples of *S. triandra* subsp. *triandra* (cluster 2) were distantly positioned from other subg. *Salix* members. *S.triandra* subsp. *bornmuelleri* were located out of cluster 2 but were only represented by a very low sample size. Both *Dim1* and *Dim2* were important in separating *S.triandra* subsp. *triandra* species from the others. Subgenus *Vetrix* members dominated cluster 6, which consisted of *S. caprea, S. cinerea*, *S. caucasica*, *S. myrsinifolia, S. pseudomedemii*, *S. amplexicaulis, S. wilhelmsiana, S.pedicellata* subsp. *pedicellata, S. purpurea* subsp. *leucodermis, S. rizeensis, S. elaeagnos, S. apoda* and *S. pentandroides.* However, there is a significant overlap with cluster 7, which includes the hybrid species *S. alba x fragilis*. Cluster 7 seems to be located in the mixed zone of subg. *Salix* and subg. *Vetrix* members, and includes both species of the subgenus *Salix* (*S. acmophylla* and *S.pentandroides*) and subgenus *Vetrix* (*S. armenorossica*, *S. viminalis*, *S. elbursensis, S. pseudodepressa* and *S. aegyptiaca*). Although all samples of *S. acmophylla* were located in the mixed zone, *S. pentandroides* samples were dispersed in both the mixed zone and in the *Vetrix* clusters. Like *S.pentandroides*, *S.amplexicaulis* samples were also nested in both clusters 6 and 7.

In Figure 5, the MCA plot reveals the first two dimensions, showing the differentiation of the two Turkish subgenera (Subg. *Salix* and *Vetrix*) based on morphological data. Each species is represented

by a high sample size in subg. *Salix* members, whilst there were only a limited number of samples for species in subg. *Vetrix* (Table 1). These results indicate that the two subgenera were almost separated within the two MCA dimensions (Figure 5). The binary data such as tree life form, leaf lanceolate shape (for subg. *Salix*) in *Dim1* and pubescence bud scale (for subg. *Vetrix*) in *Dim3* were the dominant characters in subgenera grouping. Most members of subg. *Salix* were clustered at the top-left position, whereas subg. *Vetrix* members are clustered at middle-lower positions by the *Dim1*. Additionally, there is a mixture of subg. *Vetrix* with the subg. *Salix* in the intersection zone. The species *S. triandra* subsp. *triandra* was clustered distantly at the top-right of the MCA plot, separated from the members of subg. *Salix*. Such a distinct separation (cluster 2; 2n=2x=38) from the subg. *Salix* members (2n=4x=76) and the top position of the subg. *Salix* members within the molecular tree may be due to different chromosomal rearrangements (Hamza-Babiker et al., 2009). Some limitations should be noted, however, as we only used one nrDNA region and 11 morphological characters to understand and evaluate Turkish willow species. Although further molecular phylogenetic studies will be required to clarify the taxonomic status of willows, our dataset provides the first morphological and phylogenetic analysis using advanced programs on the complex Turkish *Salix* sp.

The Role of Biogeography for Both Datasets

Biogeographically, the subg. *Salix* dispersed in the continental climate of central and southwestern Turkey, whereas subg. *Vetrix* species adapted to high latitude, altitude and the cool climate of northern Turkey (Figure 1). The clear separation of two subgenera of Turkish *Salix* species was highlighted by the molecular (12 substitutions in nrDNA *ETS*) and morphological (life form, lanceolate leaf shape and pubescence bud scale) datasets presented in this study. In the subgenera clustering, bud scales with pubescence (one of the morphological characteristics of Turkish *Salix* subg. *Vetrix*) can reduce the grazing and conserve the leaf from damage by solar radiation in habitats with high altitudes (Ehleringer and Björkman, 1978). Most of the subg. *Salix* species are characterized by tree-like life forms and lanceolate leaf shapes. The appearance of a distinct lanceolate leaf form in subg. *Salix*, which is widely distributed in Turkey, is inconsistent with taxonomists' previous morphological classifications (Davis, 1965-1988). These findings are in accordance with Skvortsov's (1999) statements that subg. *Salix* is a natural and ancient group displaying primitive characteristics, while subg. *Vetrix* includes species characterized by more advanced and recently evolved traits. The reticulate relations and high rate of polymorphism in subg. *Vetrix* also support the occurrence of recently evolved and complex relations (Hardig et al., 2010).

S. acmophylla (subg. *Salix*), naturally found in the Eastern part of Turkey, is well allied far from members of subg. *Salix* in both datasets. All *S. acmophylla* samples were gathered from Urfa and Batman (Figure 1). A potential explanation for this distant positioning might be related to the effect of the Anatolian Diagonal, which is an important geographic speciation barrier, causing taxonomic differentiation between subg. *Salix* members (Bilgin, 2011). Another interesting and distant species of subg. *Salix* is *S. pentandroides:* this species was clustered with subg. *Vetrix*, while samples from the Çoruh river and Erzurum were clustered with samples from mixed zone. Those two sampling locations varied in altitude, latitude, and climatic conditions. Since environmental variables have important impacts on *Salix* growth and natural distribution, morphological characters will be selected and expressed differently in diverse habitats (Skvortsov, 1999; Yıldırım and Kaya, 2017). Thus, *S. pentandroides* members were grouped distantly from subg. *Salix* in both datasets. *S. amplexicaulis*, a member of subg. *Vetrix* separated from subg. *Vetrix* groups for molecular and morphological data. The distant appearance of *S. amplexicaulis* may be explained by possible hybridizations with this subg. *Salix* species in mixed habitats. Therefore, we strongly suggest that *S.amplexicaulis* taxonomically need to be merged with subg. *Salix.* As only one herbarium sample represented *S. rizeensis*, more information should be obtained to evaluate the taxonomic position of this endemic species. Extensive hybridization events in *Salix* L. have resulted in intermediate forms of various morphological characters commonly observed in the hybrid species *S.alba* x *fragilis*. The hybrid was located near *S.fragilis* and *S.alba* in our molecular tree and in the mixed zone in morphological clustering, as expected.

CONCLUSION

The studied molecular gene region and morphological traits accurately reflected the taxonomic relationships in Turkish *Salix* species. These species are classed into two subgenera in regards to 12 variables sites in the external transcribed spacer of the ribosomal nuclear DNA gene region and three vegetative morphological characters. The first 3 dimensions (*Dim1*, *Dim2,* and *Dim3*) of our morphological data explained 33.3 % of the total morphometric variation. The pubescence on bud scale was discriminative for the subgenus *Vetrix* members located in high-altitude habitat, while tree-like life forms and lanceolate leaf shapes were characteristic of subgenus *Salix* members. Results from our molecular data suggest that *S. amplexicaulis*, which is currently in subg. *Vetrix*, should be merged into subg. *Salix*. Subgenus *Salix* members *S.acmophylla* and *S.pentandroides* were classified as a distinct species, in accordance with our molecular and morphological datasets, as a consequence of their biogeographical distribution in Turkey. This study provided novel molecular and morphometric findings to the poorly understood woody genera *Salix* L. and it results showed more useful information than that found in previous literature. In the Turkish *Salix* species, our molecular analysis supported the results from morphological taxonomy. A more comprehensive study covering all Turkish *Salix* species and more genomic regions is necessary to construct an accurate taxonomic classification for *Salix*.

Ethics Committee Approval: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – P.A., Z.K.; Design – P.A., Z.K.; Supervision – P.A., Z.K.; Resources – P.A., Z.K., F.Ö.D.; Materials – P.A., F.Ö.D.; Data Collection and/or Processing – P.A., B.T., F.Ö.D.; Analysis and/or Interpretation – P.A., B.T.; Literature Search – P.A., B.T.; Writing Manuscript – P.A.; Critical Review – P.A., Z.K.; Other – P.A., Z.K.

Acknowledgements: This study was funded by the Scientific and Technical Research Council of Turkey (TUBITAK) under project TOVAG

213O154 "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 supported by the Middle East Technical University (METU) under project BAP-01-08-2012-013 "Türkiye Söğüt Türlerinin Moleküler Filogenetiği". We are also grateful to Hayri Duman, Meral Avcı, Ali Dönmez, S.Tuğrul Körüklü and Tuğçe Alp for their supports and for providing herbarium materials from their GAZI, HUB and ANK herbariums.

Conflict of Interest: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

REFERENCES

- Abdi, H., Valentin, D., 2007. Multiple correspondence analysis. In N.J. Salkind (Ed.): Encyclopedia of Measurement and Statistics, Thousand Oaks (CA)Sage, pp. 651-657.
- Abdollahzadeh. A., Kazempour. O.S., Maassoumi, A. A., 2011. Molecular Phylogeny of the Genus *Salix* (Salicaceae) with an emphasis to its species in Iran. *Iranian Journal of Botany* 17(2): 244-253.
- Acar, P., 2017. Molecular Phylogenetics of Turkish *Salix* L. species (Unpublished Ph.D. Thesis, Middle East Technical University, p. 123, Ankara Turkey.
- Akgül, S ., Tunçtaner K., 2011. Selection of suitable willow clones for biomass production and growing techniques in Turkey, in: Tercer Congreso Internacional de Salicáceas en Argentina, Neuquén, Argentina.
- Argus, G.W., 1997. Infrageneric Classification of *Salix* (Salicaceae) in the New World. *Systematic Botany Monographs* 52: 1-121. **[CrossRef]**
- Arıhan, O., Güvenç, A., 2011. Studies on the anatomical structure of stems of willow (*Salix* L.) species (Salicaceae) growing in Ankara province. *Turkish Journal of Botany* 35: 535-551.
- Avcı, M., 1999. Türkiye'nin Doğal Söğütleri ve Coğrafi Dağılışları. İstanbul Üniversitesi Edebiyat Fakültesi. *Coğrafya Bölümü Coğrafya Dergisi* 7: 1-24.
- Azuma, T., Kajita, T., Yokoyama, J., Ohashi, H., 2000. Phylogenetic relationships of *Salix* (Salicaceae) based on *rbc*L sequence data. *American Journal of Botany* 87: 67-75. **[CrossRef]**
- Baldwin, B.G., Markos, S., 1998. Phylogenetic utility of the external transcribed spacer (ETS) of 18S-26S rDNA: congruence of *ETS* and *ITS* trees of Calycadenia (Compositae). *Mol Phylogenet Evol* 10(3): 449-63. **[CrossRef]**
- Barkalov, V.Y., Kozyrenko, M.M., 2014. Phylogenetic relationships of *Salix* L. subg. *Salix* species (Salicaceae) according to sequencing data of intergenic spacers of the chloroplast genome and *ITS* rDNA. *Russion Journal of Genetic* 50(8): 828-837. **[CrossRef]**
- Bilgin R., 2011. Back to the Suture: the distribution of intraspecific genetic diversity in and around Anatolia. *International Journal of Molecular Sciences* 12: 4080-4103. **[CrossRef]**
- Chen, J., Sun, H., Wen, J. Yang, Y., 2010. Molecular Phylogeny of *Salix* L. (Salicaceae) Inferred from Three Chloroplast Datasets and Its Systematic Implications. International Association for Plant Taxonomy (IAPT). *Taxon* 59(1): 29-37. **[CrossRef]**
- Davis, P.H.R., 1965-1988. in Flora of Turkey and the East Aegean Islands, Vol. 7. 1st ed.- Edinburgh: UK: Edinburgh University Press, pp. 694-716.
- Degirmenci, F.O., Acar P., Kaya, Z., 2019. Consequences of habitat fragmentation on genetic diversity and structure of *Salix alba* L. populations in two major river systems of Turkey. *Tree Genetics & Genomes* 15: 59. **[CrossRef]**
- Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* 7(1): 214. **[CrossRef]**
- Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11-15.
- Ehleringer, J.R., Björkman, O., 1978. A Comparison of Photosynthetic Characteristics of Encelia Species Possessing Glabrous and Pubescent Leaves. *Plant Physiology* 62: 185-190. **[CrossRef]**
- Güner, A., 2000. *Salix* L.in: Güner, A., Özhatay, N., Ekim, T., Başer, K.H.C. (eds.), Flora of Turkey and the East Aegean Islands. Vol.11.-Edinburgh: Edinburgh University Press, pp. 216-217.
- Hamza-Babiker, N., Berthold, H., Glössl, J., Arnold, C., 2009. Chloroplast DNA identification of eight closely related European *Salix* species. *Austrian Journal of Forest Science* 126: 175-193.
- Hardig, T.M., Anttila, C.K., Brunsfeld, S.J., 2010. A Phylogenetic Analysis of *Salix* (Salicaceae) Based on *mat*K and Ribosomal DNA Sequence Data. *Journal of Botany* 2010: 1-12. **[CrossRef]**
- Herrera, S., 2006. Bonkers about biofuels. *Nature Biotechnology* 24(7): 755-660. **[CrossRef]**
- Kuzovkina, Y.A., Quigley, M.F., 2005. Willows beyond wetlands: uses of *Salix* L. species for environmental projects. *Water, Air & Soil Pollution* 162: 183-204. **[CrossRef]**
- Lauren-Moreau, A., Pitre, E.F., Argus, G.W., Labrecque, M., Brouillet, L., 2015. Phylogenetic Relationships of American Willows (*Salix* L., Salicaceae). *PLoS One* 10(9): e0138963. **[CrossRef]**
- Librado, P., Rozas, J., 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25(11): 1451-1452. **[CrossRef]**
- Rambaut, A., 2016. Figtree 1.4.3. Available from: http://tree.bio.ed.ac.uk/software/figtree/. (Accessed: 01 August 2018).
- R Core Team, 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available from: http://www.R-project.org/.
- Skvortsov, A.K., 1999. Willows of Russia and adjacent countries: Taxonomical and Geographical Revision (transl from: Skvortsov A.K.1968: Willows of the USSR: Taxonomic and Geographic Revision. Nauka, Moscow), ed: Zinovjev A.G., Argus G.W., Tahvanainen, J. & Roininen, H., Joensuu Unviersity, Joensuu, p.307.
- Patterson, J., Chamberlain, B., Thayer, D., 2004-2006. Finch TV Version 1.4.0.
- Suda, Y., Argus, G.W., 1968. Chromosome numbers in some North American *Salix*. *Brittonia* 20: 191-197. **[CrossRef]**
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30(12): 2725-9. **[CrossRef]**
- Terzioğlu, S., Serdar, B., Karaköse, M., Coşkunçelebi, K., Gültepe, M., 2014. New data on *Salix anatolica* (Salicaceae) endemic to Turkey. *Phytotaxa* 167: 111-118. **[CrossRef]**
- Vermerris, W., 2008. Genetic Improvement of Bioenergy Crops. Springer, pp. 347-362. **[CrossRef]**
- Weeks, A., Daly, D.C., Simpson, B.B., 2004. The phylogenetic history and biogeography of frankincense and myrrh family (Burseraceae) based on nuclear and chloroplast sequence data. *Molecular Phylogenetics and Evolution* 35: 85-101. **[CrossRef]**
- Wu, J., Nyman, T., Wang, D., Argus, G.W., Yang, Y., Chen, J., 2015. Phylogeny of *Salix* subgenus *Salix* s.l. (Salicaceae): delimitation biogeography and reticulate evolution. *BMC Evolutionary Biology* 15: 31. **[CrossRef]**
- Yıldırım, K., Kaya, Z., 2017. Gene regulation network behind drought escape, avoidance and tolerance strategies in black poplar (*Populus nigra* L.). *Plant Physiology and Biochemistry* 115: 183-199. **[CrossRef]**
- Zielinski, J., Tomaszevski, D., 2007. *Salix anatolica* (Salicaceae), a new species from Turkey. *Annales Botanici Fennici* 45: 386-388.**[CrossRef]**